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CHAPTER 72

Herpes Simplex Viruses and Their Replication

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> . . . paradoxically it is much easier for people to adapt the observed facts dialectically to the ruling paradigm than to renounce the ruling paradigm in response to possible new interpretations of the facts.
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> Carlo M. Cipolla in *Miasmas and Disease. Public Health and the Environment in the Pre-Industrial Age*. p. 6. Yale University Press, 1992.

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Herpes simplex viruses (HSV) were the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses. Their attractions are their biologic properties, particularly their abilities to cause a variety of infections, to remain latent in their host for life, and to be reactivated to cause lesions at or near the site of initial infection. They serve as models and tools for the study of translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, and a myriad of other biologic problems, both general to viruses and specific to HSV.

For years, their size and complexity served as formidable obstacles to intensive research. More than 40 years passed from the time of their isolation until Schneweiss (597) demonstrated that there were in fact two serotypes, HSV-1 and HSV-2, whose formal designations under International Conference for Taxonomy of Viruses (ICTV): rules are now human herpesviruses 1 and 2 (556). Not until 1961 were practical plaque assays published (564), and only much later were the genome sizes and the extent of homology between these two viruses reported. This chapter recounts well established facts, but its main emphasis is on burning issues, the problems whose time has come.

Virology conserves three myths. The first is that research on a virus reaches its peak when the number of investigators approaches the number of nucleotides in its genome. This formula calls for 152,000+ investigators, one for each base pair (309,402). In orders of magnitude, we are close but not yet there. There are times when we think almost that many bodies will be needed to unravel all the mysteries of these viruses. The second myth is that virologists repeat the same experiment over and over again. As with all myths, there may be a grain of truth here. In wading through the mass of papers published in the past decade, it was instructive to see how many times the same phenomenon was published or rediscovered time and time again under the same or different name.

Lastly, the oft-made statement that science simplifies knowledge is patently a myth. The falsehood of this premise is attested by this chapter. What should have been a simple update of the chapter published in the second edition of *Field's Virology* unraveled complexities far beyond our expectations.

VIRION STRUCTURE

The HSV virion consists of four elements: (i) an electron-opaque core, (ii) an icosadeltahedral capsid surrounding the core, (iii) an amorphous tegument surrounding the capsid, and (iv) an outer envelope exhibiting spikes on its surface (557).

The dry masses of herpes simplex virions, full nucleocapsids, empty nucleocapsids, and cores were calculated from permeability of virions to electrons to be $13.33 \pm 2.56 \times 10^{-16}$ g, $7.55 \pm 1.11 \times 10^{-16}$ g, $5.22 \pm 1.10 \times 10^{-16}$ g, and $2.07 \pm 0.95 \times 10^{-16}$ g, respectively (350). The average mass ratios of the virion, full capsid, and core to DNA are 8.1, 4.6, and 1.25, respectively. The experimentally derived ratio of virus mass to DNA is 10.73 ± 0.96, from which it has been calculated that the virion contains 19.4×10^{-16} g of protein (242). A similar value was derived from counts of virions in purified virus preparations (R. W. Honess and B. Roizman, unpublished data), although the error in the these determinations was higher. This ratio was used in the calculation of the polypeptide content of HSV virions by Heine et al. (242).

Virion Polypeptides

Early studies on purified HSV-1 virions suggested that they contain 30+ proteins designated as virion polypeptides (VP) and given serial numbers (242,643). All of the virion proteins were made after infection, and no host proteins could be detected in purified virion preparations. Of the approximately 30 known and another ten suspected virion proteins (Table 1), at least 9 are on the surface of the virion (accessible to antibody) and at least ten are glycosylated. The glycoproteins are gB (VP7 and VP8.5), gC (VP8), gD (VP17 and VP18) and gE (VP12.3 and VP12.6), gG, gH, gI, gK, gL, and gM. Another small glycoprotein, given the designation gJ, was predicted by DNA sequence analyses. Virion envelopes also contain at least two (U_L20 and U_L34) and possibly more (U_L24 and U_L43??) nonglycosylated intrinsic membrane proteins. Stannard et al. (653) reported that spikes projecting from envelopes are, as was expected, the viral glycoproteins, and that the latter were nonrandomly distributed.

Gibson and Roizman (213,215) described three kinds of capsids, i.e., those that lack DNA and were never enveloped (type A), those that contain DNA and were never enveloped (type B), and those that contain DNA and were obtained by deenveloping intact virions (type C). In the current nomenclature, the term A capsid refers to capsids without an internal toroidal structure which is thought to act as scaffolding for packaging of DNA, those with scaffolding but without DNA are designated as B capsids, and those with DNA have been designated as the C capsids (Fig. 1).

The empty (A) capsids consist of four proteins, i.e., VP5 (U_L19), VP19C (U_L38), VP23 (U_L38), and a smaller M_r

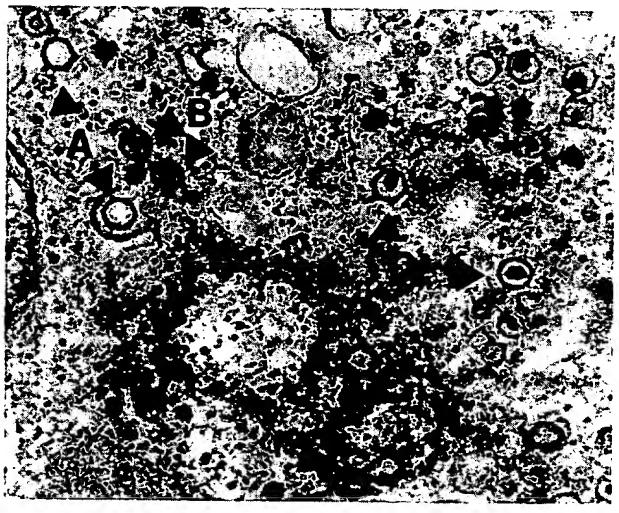

FIG. 1. This section of a nucleus of a Vero cell culture harvested 18 hr postinfection with HSV-1(F). **A:** Empty capsids devoid of scaffolding protein. **B:** Capsids containing internal proteins arranged as a ring and presumed to be the scaffolding proteins. **C:** Capsids containing DNA.

TABLE 1. *Herpes simplex virus genes, their products, and their functions*

Gene or Transcriptional Unit	Designation of Protein	Dispensable for Replication in Cell Culture	Regulation: Kinetic Group	Function of Gene Product
$\gamma_1$34.5	ICP34.5	Y	γ_1	Deletion mutants fail to replicate in central nervous system of mice. In human cells in culture, the $\gamma_1$34.5$^-$ viruses fail to preclude programmed cell death initiated by complete cessation of viral protein synthesis. Carboxyl terminus homologous to the corresponding domain of the GADD34 and MyD116 proteins.
ORF P	ORF P	Y	??	Open reading frame is antisense to $\gamma_1$34.5. The gene is expressed under conditions in which ICP4 is nonfunctional.
ORF O	ORF O	Y	?	Open reading frame partially antisense to the $\gamma_1$34.5 gene. The protein is expressed under conditions in which ICP4 is not functional.
α0	ICP0	Y	α	Nucleotidylylated, phosphoprotein, promiscuous *trans*-activator of genes introduced by transfection or infection; optimal activity requires presence of ICP4. Deletion mutants debilitated with respect to replication at low multiplicities of infection.
U_L1	gL	N	γ	Forms complex with gH. Complex required for transport of both proteins to plasma membrane and for viral entry mediated by gH. Contains *syn* locus.
U_L2		Y	β	Uracil DNA glycosylase.
U_L3		Y	Unknown	Unknown function. Identified in HSV-2 as a nuclear phosphoprotein. Protein has nuclear localization signal and is unglycosylated. Reported to localize to perinuclear region early and to the nucleus late in infection.
U_L4		Y	γ_2	Identified three protein species: the M_r 60k species is found in virions and light particles (19).
U_L5		N	β	Forms complex with U_L8 and U_L52 proteins.
U_L6		N	Unknown	Virion protein; required for DNA cleavage-packaging.
U_L7		N	Unknown	Unknown.
U_L8		N	β	Forms complex with U_L5 and U_L52, acts as a primase and expresses helicase activity in the presence of U_L9 protein. Stabilizes interaction between primers and DNA template.
U_L9		N	γ(?)	Binds to origins of DNA synthesis in sequence-specific (origin) fashion; carries out helicase and ATPase activities.
U_L10	gM	Y	γ	Glycoprotein present in virions and plasma membranes.
U_L11		Y	γ(?)	Myristylated protein; necessary for efficient capsid envelopment and exocytosis.
U_L12		Y	β	Alkaline exonuclease (DNAse)—involved in viral nucleic acid metabolism; reported to localize in nucleoli and in virally induced nuclear dense bodies and to bind to a sequence along with other unidentified proteins. Complex may be involved in cleavage-packaging of viral DNA.
U_L13		Y	γ	Nuclear protein suspected of being a protein kinase; required for phosphorylation of ICP22, VHS.
U_L14		N	Unknown	Unknown.
U_L15		N	γ	ts mutant DNA+. Two exons; protein required for packaging of DNA.
U_L16		Y	Unknown	Located within intron of U_L15.
U_L17		N	γ	Located within intron of U_L15.
U_L18	VP23	N	γ	Capsid protein required for capsid formation and cleavage-packaging of replicated viral DNA.
U_L19	VP5, ICP5	N	γ_1	Major capsid protein.
U_L20		Y	γ	Intrinsic membrane protein necessary for viral exocytosis, particularly in cells in which the Golgi apparatus is fragmented and dispersed.
U_L20.5	U_L20.5	Y	γ_2	Unknown.
U_L21		Y	Unknown	Nucleotidylylated phosphoprotein; unknown function.
U_L22	gH	N	γ_2	Forms complex with gL (see above). Appears to play a role in entry, egress, and cell-cell spread.

TABLE 1. *Continued.*

Gene or Transcriptional Unit	Designation of Protein	Dispensable for Replication in Cell Culture	Regulation: Kinetic Group	Function of Gene Product
U_L23	ICP36	Y	β	Thymidine kinase, more properly a nucleoside kinase.
U_L24		Y	γ	*syn*⁻ locus; membrane-associated protein?
U_L25		N	γ	Virion protein reported to be required for cleavage-packaging of viral DNA.
U_L26		N	γ	Serine protease; substrates are U_L26 protein and $U_L26.5$ (ICP35 capsid proteins)—VP21, VP22a, and VP24 capsid proteins are all products of the self-cleavage of U_L26; VP21 suggested to be C-terminal portion of U_L26 after cleavage release of VP24. VP24 contains protease domain from N-terminal portion of U_L26.
$U_L26.5$	ICP35	N	γ	Substrate of U_L26 protease; the precursor, ICP35b,c is cleaved to ICP35e,f. The protein is unique to B capsids and forms its inner core or scaffolding. On packaging of DNA, VP22A is removed from capsid shell.
U_L27	gB, VP7	N	γ_1	Glycoprotein forms a dimer and induces neutralizing antibody. Required for viral entry. A syn locus maps to the carboxyl terminus.
U_L28	ICP18.5	N	γ	M_r 87- to 95-k protein required for DNA cleavage-packaging.
U_L29	ICP8	N	β	Binds singled-stranded DNA cooperatively, required for viral DNA replication. Mutants are DNA⁻. Expression of early and late genes may be affected positively or negatively by the function of ICP8. ICP8 binds to single-stranded DNA and facilitates renaturation of complementary strands of DNA, homologous pairing, and strand transfer.
U_L30		N	β	DNA polymerase; forms complex with C-terminal 247 amino acids of U_L42 protein.
U_L31		N	γ_2	Nucleotidylylated phosphoprotein, cofractionates with nuclear matrix.
U_L32		N	γ_2	ts mutant is deficient in DNA packaging.
U_L33		N	Unknown	DNA packaging; necessary for assembly of capsids containing DNA.
U_L34		N	Unknown	Abundant nonglycosylated, membrane-associatad, virion protein phosphorylated by protein kinase U_S3.
U_L35	VP26	N	γ_2	Basic phosphorylated capsid protein.
U_L36	ICP1-2	N	γ_2	Virion tegument phosphoprotein. In cells infected with ts mutant at nonpermissive temperatures, DNA is not released from capsids at nuclear pores. Reported to form complex with M_r 140k protein that binds a sequence DNA. May be involved in cleavage and/or packaging of newly synthesized viral DNA.
U_L37	ICP32	N	γ	Cytoplasmic phosphoprotein; in presence of ICP8, it is transported to nucleus and associates with DNA, but phosphorylation is not dependent on ICP8.
U_L38	VP19C	N	γ_2	Capsid assembly protein, binds DNA, and may be involved in anchoring DNA in the capsid.
U_L39	ICP6	Y	β	Large subunit of ribonucleotide reductase. Autophosphorylates via unique N-terminus but does not transphosphorylate. HSV-2 homolog can be transphosphorylated.
U_L40		Y	β	Small subunit of ribonucleotide reductase.
U_L41	VHS	Y	γ	Causes nonspecific degradation of mRNA and shut off of macromolecular synthesis after infection. Exists as two phosphorylated species with M_r of 58 and 59.5 k.
U_L42		N	β	Double-stranded DNA-binding protein, binds to and increases processivity of DNA polymerase.
U_L43		Y	Unknown	Amino acid sequence predicts membrane-associated protein.
U_L44	gC, VP8	Y	γ_2	Glycoprotein involved in cell attachment; required for attachment to the apical surface of polarized MDCK cells.
U_L45		Y	γ_2	Encodes a M_r 18k protein of unknown function.
U_L46	VP11/12	Y	γ	Tegument phosphoprotein reported to modulate the activity of U_L48 (αTIF) protein.

TABLE 1. *Continued.*

Gene or Transcriptional Unit	Designation of Protein	Dispensable for Replication in Cell Culture	Regulation: Kinetic Group	Function of Gene Product
U_L47	VP13/14	Y	γ_2	Nucleotidylylated tegument phosphoprotein reported to modulate the activity of U_L48 (αTIF) protein.
U_L48	VP16, ICP25, αTIF	N	γ	Tegument protein, induces α genes by interacting with host proteins, including Oct-1. The complex binds to specific sequences with the consensus GyATGnTAATGArATTCyTTGnGGG-NC.
U_L49		N	γ	Nucleotidylylated mono(ADP-ribosyl)ated tegument phosphoprotein.
$U_L49.5$		N	γ_2	Sequence predicts a M_r 12k membrane-associated protein.
U_L50		Y	β	dUTPase.
U_L51		Y	γ	Unknown.
U_L52		N	β	Component of the helicase/primase complex.
U_L53	gK	N	γ	Glycoprotein required for efficient viral exocytosis; contains syn locus.
U_L54	α27, ICP27	N	α	Nucleotidylylated, multifunctional regulatory protein required for late gene expression. The protein negatively regulates early genes. It was reported to cause the redistribution of snRNPs and to inhibit RNA splicing.
U_L55		Y	Unknown	Unknown.
U_L56		Y	Unknown	Nuclear, virion-associated protein of unknown function.
α4	ICP4	N	α	Nucleotidylylated, poly(ADP-ribosyl)ated, phosphoprotein regulates positively most β and γ genes and negatively itself, OFRF-P and the α0 genes. It binds to DNA in sequence-specific fashion.
U_S1	α22, ICP22	Y	α	Nucleotidylylated regulatory protein, phosphorylated by U_L13 protein kinase, required for optimal expression of ICP0 and of a subset of γ proteins.
$U_S1.5$	$U_S1.5$	Y	α	Gene 31 coterminal with U_S1. Not required for optimal expression of α0 or late (γ) genes.
U_S2		Y	Unknown	Unknown.
U_S3		Y	β	Protein kinase; major substrate is U_L34 protein.
U_S4	gG	Y	γ	Glycoprotein involved in entry, egress, and spread from cell to cell.
U_S5	gJ(?)	Y	Unknown	Sequence predicts glycoprotein.
U_S6	gD	N	γ_1	Glycoprotein required for postattachment entry of virus into cells.
U_S7	VP17/18, gI	Y	γ	gI and gE glycoproteins form complex for transport to plasma membrane and also to constitute a high-affinity Fc receptor. gI is required for basolateral spread of virus in polarized cells.
U_S8	gE	Y	γ_2	Fc receptor; involved in basolateral spread of virus in polarized cells.
$U_S8.5$		Y	β or γ_1	Unknown.
U_S9		Y	Unknown	Tegument protein.
U_S10		Y	Unknown	Tegument protein.
U_S11		Y	γ_2	Abundant virion tegument protein binds to U_L34 mRNA in sequence and conformation specific fashion and acts as an antiattenuation factor; binds to the 60S ribosomal subunit and localizes in the nucleolus.
U_S12	α47, ICP47	Y	α	Blocks presentation of viral peptides to MHCI restricted cells.
Ori_STU	Ori_SRNA	Y	γ_2	RNA transcribed across S component origins of DNA synthesis. Most probably not translated and function is not known.
LATU	LATs	Y	?	Transcripts, some spliced, from the inverted repeat sequences flanking U_L sequences. The function of these transcripts is not known.

syn locus, mutation causes infected cells to fuse; tegument, a component of the virion located between the envelope and the capsid; Ori_STU, transcriptional unit comprising the domain of the S component origins of DNA synthesis; LATU, latency-associated transcriptional units; αTIF, α *trans*-inducing factor; HSV, herpes simplex virus; dUTP, deoxyuridine triphosphate; mRNA, messenger RNA.

12,000 protein described subsequently (105) and often referred to as VP26 (U_L35) (130,486). VP5 was estimated to be present in ratios of 850 to 1,000 per virion, i.e., approximately six per hexameric capsomere (242,557,703), but Schrag et al. (598) suggested that VP5 is a component of both pentameric and hexameric capsomeres. VP19C and VP5 appear to be linked by disulfide bond (758) and are present in approximately similar ratios per virion (242). Braun et al. (58) showed that VP19C, identified as the infected cell protein (ICP) number 32, bound to DNA and may be involved in anchoring the viral DNA in the capsid. The HSV-2 counterpart has also been mapped (752). The A-type capsids may be a decay product not in the pathway of virion maturation (618). B-type capsids differ from the A type in that they contain three additional proteins, i.e., VP21, VP22a, and VP24. VP22a corresponds to ICP35e-f, the product of the open reading frame $U_L26.5$ cleaved at the carboxyl terminus by the protease (370,371). VP21 corresponds to ICP35b (amino acids 248–610) of the protease Pra, the nascent U_L26 gene product. VP24 corresponds to the N-terminal domain (codons 1 through 247) of Pra designated as Prn, the smallest form of the protease encoded by the open reading frame U_L26. Pra is cleaved by the autologous protease between Ala^{247} and Ser^{248} and between Ala^{610} and Ser^{611} (144,368). The ICP35 family of proteins described by Braun et al. (60) plays a vital role in capsid assembly and encapsidation of viral DNA (205,507,538, 618). Gibson and Roizman (215) suggested that VP21 is an internal capsid protein. Newcomb and Brown (451,452) demonstrated that VP19 and VP23 are on the surface of the capsid and could form a network of fibers located between capsomeres (intercapsomeric fibers) and that VP22a is in the interior of the capsid and forms a ringlike structure which is quantitatively removed by 2.0 M guanidine hydrochloride.

Type C capsids were reported to contain a smaller protein—VP22—but not VP22a, and it has been suggested that the proteins are related (213,215). Sherman and Bachenheimer (618) and Rixon et al. (538) suggested that the VP22 found in the C-type capsids may not be related to VP22a. Depending on the procedure used for stripping the envelope, the C-type capsids may contain variable amounts of tegument proteins. Schrag et al. (598) have reported an elegant model of the HSV-1 capsid.

It should be noted that, in the interval between 1965 and 1974, a large number of articles dealt with the structure and morphogenesis of herpesvirus capsids; a list of citations and review of that literature was published by Roizman and Furlong (557). On the basis of electron microscopic appearance of the capsid and the core, capsids form eight groups (557). Although the various forms probably reflect different stages of capsid assembly, the reagents necessary to relate the various forms to specific proteins are only now becoming available.

The space between the undersurface of the envelope and the surface of the capsid was designated as the tegument (557); it contains the rest of the virion proteins. The most notable of the proteins associated with the space between the underside of the envelope and the capsid are the α-*trans*-inducing factor (αTIF; ICP25; VP16), VP11-12 (U_L46), VP13-14, (U_L47), the virion host shut off (VHS) protein (U_L41), the product of the U_S11 gene, and a very large protein (VP1-2) associated with a complex which binds to the terminal *a* sequence of the viral genome (33,96, 530,574,573,755). Extensive discussion of the various types of capsids and virions can be found elsewhere (557).

Recently, several publications have reported on the production of "light particles" devoid of DNA. These particles consist of enveloped tegumentlike structures (417,536, 678). They appear to contain, in addition, other nonstructural proteins previously associated with virions (e.g., ICP4) (750). Little is known of their synthesis beyond the facts that they do not appear to be uniform in size and that the capsid is not an essential trigger for envelopment or egress.

Viral DNA

Like other herpesvirus DNAs, the bulk of packaged HSV DNA is linear and double stranded (36,309,488). In the virion, HSV DNA is packaged in the form of a toroid (202). The ends of the genome are probably held together or are in close proximity inasmuch as a small fraction of the packaged DNA appears to be circular and a large fraction of the linear DNA circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells (489). DNA extracted from virions contains ribonucleotides, nicks, and gaps (42,197,736).

The HSV genome is approximately 150 kbp, with a G+C content of 68% for HSV-1 and 69% for HSV-2 (36,309,402). It consists of two covalently linked components, designated as L (long) and S (short) (Fig. 2). Each component consists of unique sequences bracketed by inverted repeats (604; 695). The repeats of the L component are designated *ab* and *b′a′*; those of the S component are *a′c′* and *ca* (Fig. 2) (706). The number of *a* sequence repeats at the L-S junction and at the L terminus is variable; the HSV genome can then be represented as

$$a_L a_n b\text{-}U_L\text{-}b'a'_m c'\text{-}U_S\text{-}ca_S$$

where a_L and a_S are terminal sequences with unique properties described below, and a_n and a_m are terminal *a* sequences directly repeated zero or more times (n) or present in one to many copies (m) (372,549,550,706,710). The structure of the *a* sequence is highly conserved but consists of a variable number of repeat elements. In the HSV-1(F) strain, the *a* sequence consists of a 20-bp direct repeat (DR1), a 65-bp unique sequence (U_b), a 12-bp direct repeat (DR2) present in 19 to 23 copies per *a* sequence, a 37-bp direct repeat (DR4) present in two to three copies, a 58-bp unique sequence (U_c), and a final copy of DR1 (436,438). The size of the *a* sequence varies from strain to

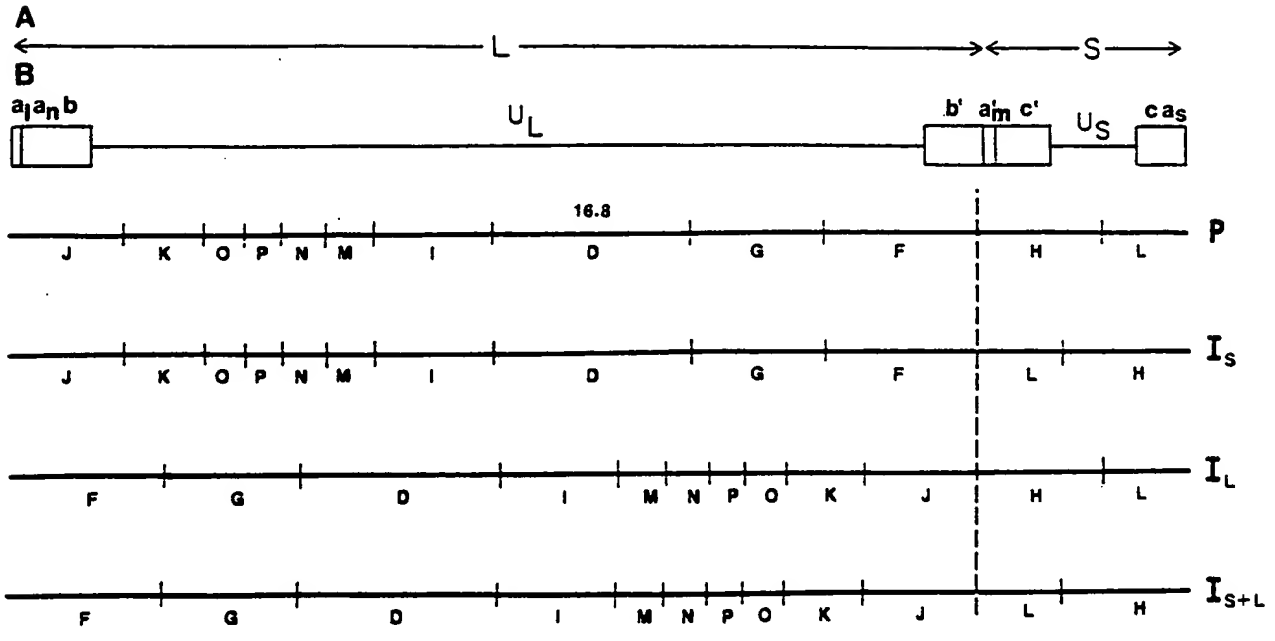

FIG. 2. Schematic representation of the arrangement of DNA sequences in the HSV genome. **A:** The domains of the L and S components are denoted by the *arrows*. The second line shows the unique sequences (*thin lines*) flanked by the inverted repeats (*boxes*). The letters above the second line designate the terminal *a* sequence of the L component (a_L), a variable (n) number of additional *a* sequences, the *b* sequence, the unique sequence of the L component (U_L), the repetitions of the *b* sequence and of a variable (m) number of *a* sequences (a_m), the inverted *c* sequence, the unique sequence of the S component (U_S), and finally the terminal a sequence (a_S) of the S component. **B:** The HindIII restriction endonuclease map of HSV-1(F) strain for the P, I_S, I_L, and I_{SL} isomers of the DNA. Note that, because HindIII does not cleave within the inverted repeat sequences, there are four terminal fragments and four fragments spanning the internal inverted repeats in concentrations of 0.5 and 0.25 M, respectively, relative to the concentration of the viral DNA.

strain, reflecting in part the number of copies of DR2 and DR4. The structure of the HSV-1(F) *a* sequence can be represented as

$$DR1\text{-}U_b\text{-}DR2_n\text{-}DR4_m\text{-}U_c\text{-}DR1$$

with adjacent *a* sequences sharing the intervening DR1. Linear virion DNA contains asymmetric terminal *a* sequence ends. The terminal *a* sequence of the L component (a_L) contains a truncated DR1 with 18 bp and one 3′ nucleotide extension, whereas the terminal *a* sequence of the S component (a_S) ends with a DR1 containing only 1 bp and one 3′ overhanging nucleotide (436). The two truncated DR1 sequences form one complete DR1 upon circularization.

The L and S components of HSV can invert relative to one another, yielding four linear isomers (Fig. 2) (135, 239). The isomers have been designated as P (prototype), I_L (inversion of the L component), I_S (inversion of the S component), and I_{SL} (inversion of both S and L components) (239,441,442).

The first evidence for the repetition of terminal sequences in inverted orientation was based on electron microscopic studies of denatured HSV-1 DNA allowed to self-anneal (615). Electron microscopic analyses of denatured molecules allowed to self-anneal and of partial denaturation profiles of HSV DNA revealed that the terminal repeats are also repeated internally and that the repeats flanking the L component differ from those of the S component in size and sequence arrangement (706). The demonstration that restriction endonucleases which cleave outside the inverted repeats yield four terminal 0.5 M fragments and four L-S component junction fragments that are 0.25 M (Fig. 2) (239), as well as analyses of the partial denaturation profiles of Wadsworth et al. (706), supported the conclusion that the L and S components can invert relative to each other.

The internal inverted repeat sequences are not essential for growth of the virus in cell culture; mutants from which portions of unique sequences and most of the internal inverted repeats have been deleted have been obtained in all four arrangements of HSV DNA (286,490). The genomes of these mutants do not invert; each is frozen in one arrangement of the L and S components, but all retain their viability in cell culture.

Other Constituents

Polyamines

The search for polyamines in the virion evolved from the observations that HSV capsid assembly requires the addition of arginine to the medium (388,683) and that the capsid does not contain highly basic proteins that would neutralize viral DNA for proper folding inside the capsid. Highly purified virions contain the polyamines spermidine and spermine in a nearly constant ratio of 1.6 ± 0.2:1 or approximately 70,000 molecules of spermidine and 40,000 molecules of spermine per virion (214,216). The polyamines appear to be tightly bound and cannot be exchanged with exogenously added labeled polyamines. Disruption of the envelope with nonionic detergents and urea removed the spermidine but not the spermine. The spermine contained in the virion is sufficient to neutralize approximately 40% of the DNA phosphate (214). Parenthetically, proteins have been noted in association with the toroidal structure (202) in the capsid. and a capsid protein has been reported to bind DNA (58).

The compartmentalization of spermine and spermidine may reflect the distribution of polyamines in the infected cell. It is of interest to note that, after infection, the conversion of ornithine to putrescine appears to be blocked, but the synthesis of spermine and spermidine does not appear to be affected (214).

Lipids

It has been assumed that HSV acquires the envelope lipids from its host. Little is known of the composition of the lipid in the envelopes. The hypothesis that it is determined by its host was supported by the observation that the buoyant density of the virus was host cell-dependent on serial passage of HSV-1 alternately in HEp-2 and chick embryo cells (644). Since the envelope is derived from cellular membranes, it has been assumed that the viral envelope and cellular membranes contain similar or identical lipids. Recent studies suggest that the virion lipids are similar to those of cytoplasmic membranes and different from those of nuclear membranes (700).

HSV Polymorphism

Intertypic Variation

Although the genetic maps of HSV-1 and HSV-2 are largely colinear, they differ in restriction endonuclease cleavage sites and in the apparent sizes of viral proteins. Thus, the initial locations of viral genes on the linear map of HSV genomes were based on analyses of HSV-1 × HSV-2 recombinants and took advantage of the intertypic differences in the sizes of the proteins and the locations of restriction endonuclease cleavage sites (390,441,442,508).

Intratypic Variation

The first evidence of intratypic polymorphism emerged from studies of virion structural proteins and indicated that nonglycosylated proteins vary sufficiently in electrophoretic mobility to be used as strain markers (482). Although specimens from epidemiologically related individuals appeared to yield similar electrophoretic profiles, the usefulness of virion proteins as markers for molecular epidemiologic studies was limited by the effort required to purify virions for such analyses.

At the DNA level, differences between HSV-1 strains appear to result from base substitutions, which may add or eliminate a restriction endonuclease cleavage site and, on occasion, change an amino acid, or variability in the number of repeated sequences present in a number of regions of the genome, e.g., $\gamma_1$34.5, U_S11, and so forth (95,540). The restriction endonuclease cleavage patterns of a given strain are relatively stable, while the number of repeats are not (64,238,555,692). Thus, no changes in restriction endonuclease patterns were noted in isolates from the same individual over an interval of 13 years or in genomes of an HSV-1 strain passaged serially numerous times in cell culture. However, restriction endonuclease site polymorphism was readily noted in isolates from epidemiologically unrelated individuals (232,570). On the basis of these properties, restriction endonuclease site polymorphism was used in several epidemiologic studies of HSV transmission in the human population (64,555,570), and blind restriction endonuclease analysis of virus isolates has been used to trace the spread of infection from patients to hospital personnel (62), from patient to patient (366), and from hospital personnel to patient (7,63). Recently, Sakaoka et al. (587) reported on clustering of divergent sites along geographically and racially distinct areas. The conclusion that "the evolution of HSV-1 may be host dependent" is an aggressive interpretation of the data; a more conservative interpretation is that random mutations were conserved and dispersed in different populations.

VIRAL REPLICATION

General Pattern of Replication

It is convenient to begin this section on viral replication with a bird's-eye view of the major events (Fig. 3).

To initiate infection, the virus must attach to cell surface receptors. Fusion of the envelope with the plasma membrane rapidly follows the initial attachment. The deenveloped capsid is then transported to the nuclear pores where DNA is released into the nucleus.

Transcription, replication of viral DNA, and assembly of new capsids take place in the nucleus (Figs. 3 and 4).

Viral DNA is transcribed throughout the reproductive cycle by host RNA polymerase II, but with the participation of viral factors at all stages of infection. The syn-

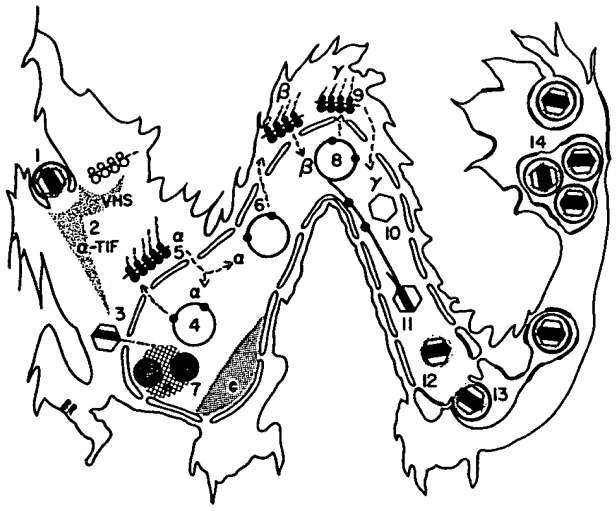

FIG. 3. Schematic representation of the replication of HSV in susceptible cells. **1:** The virus initiates infection by the fusion of the viral envelope with the plasma membrane following attachment to the cell surface. **2:** Fusion of the membranes releases two proteins from the virion. VHS shuts off protein synthesis (broken RNA in open polyribosomes). α-TIF is transported to the nucleus. **3:** The capsid is transported to the nuclear pore where viral DNA is released into the nucleus and immediately circularizes. **4:** The transcription of α genes by cellular enzymes is induced by α-TIF. **5:** The 5 αmRNAs are transported into the cytoplasm and translated (filled polyribosome); the proteins are transported into the nucleus. **6:** A new round of transcription results in the synthesis of β proteins. **7:** At this stage in the infection, the chromatin (*c*) is degraded and displaced toward the nuclear membrane, whereas the nucleoli (*round hatched structures*) become disaggregated. **8:** Viral DNA is replicated by a rolling circle mechanism, which yields head-to-tail concatemers of unit-length viral DNA. **9:** A new round of transcription/translation yields the γ proteins, consisting primarily of structural proteins of the virus. **10:** The capsid proteins form empty capsids. **11:** Unit-length viral DNA is cleaved from concatemers and packaged into the preformed capsids. **12:** Capsids containing viral DNA acquire a new protein. **13:** Viral glycoproteins and tegument proteins accumulate and form patches in cellular membranes. The capsids containing DNA and the additional protein attach to the underside of the membrane patches containing viral proteins and are enveloped. **14:** The enveloped proteins accumulate in the endoplasmic reticulum and are transported into the extracellular space.

thesis of viral gene products is tightly regulated (Fig. 5). Viral gene expression is coordinately regulated and sequentially ordered in a cascade fashion. The gene products studied to date form at least five groups on the basis of both transcriptional and posttranscriptional regulation (Fig. 5).

Several of the gene products are enzymes and DNA-binding proteins involved in viral DNA replication. The bulk of viral DNA is synthesized by a rolling circle mechanism, yielding concatemers which are cleaved into monomers and packaged into capsids.

Assembly occurs in stages; after packaging of DNA into preassembled capsids, the virus matures and acquires infectivity by budding through the inner lamella of the nuclear membrane (Figs. 3 and 6). In fully permissive tissue culture cells, the entire process takes approximately 18 to 20 hr.

Initial Stages of Infection

The available information on events preceding the transcription of viral genes is still fragmentary. The central issue is that two of the initial events, attachment to the cell surface and fusion of the viral envelope with the plasma membrane, must necessarily involve viral surface proteins. Of the ten known HSV membrane glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM) (19,192, 271,529,640), five (gC, gE, gG, gI, and gM) are dispensable in most cells in culture, both for entry into and egress from cells (22,242,375,376,377). The predicted product of the U_s5 gene, gJ, has not been shown to be present in virions, and its posttranslational modification or function has not been published.

Attachment

Both HSV-1 and HSV-2 are readily detected on the surface of cells, particularly juxtaposed to coated pits of cells exposed for a brief interval to infectious virus (Fig. 7). Attempts to find cultured mammalian cells lacking receptors have not been successful, leaving the species specificity of natural infection by this virus a mystery: only chimpanzees, other than humans, are "naturally" infected with this virus (399).

Numerous studies have been unable to assign the responsibility for attachment to the cell surface to a sole viral glycoprotein. The genes encoding each of the HSV-1 glycoproteins except gK have been deleted individually from the viral genome, and each of those viruses is able to attach to and infect nonpolarized epithelial cells (22,66,83, 190,289,290,375,376,575). The reason for this became apparent as data indicated that HSV can utilize more than one attachment pathway.

The apparent lack of a single viral protein-cell surface receptor interaction for HSV is in part due to the use for most of the attachment studies of nonpolarized, continuous cell lines. During the course of its normal life cycle, HSV must infect and replicate in two different cell types: epithelial cells and neurons. These cells are quite different from one another and also, *in vivo*, highly polarized; they sort membrane and secreted proteins to one surface or another. In the case of epithelial cells, membrane proteins are sorted to the apical or basal surfaces (77,547,548); in neurons, they are sorted to axons or dendrites (148,149). The virus therefore must be able to attach to and infect at least three very different types of membranes. For example, in nonpolarized cells, gC is dispensable for viral attachment and replication (242). The first evidence that gC was in-

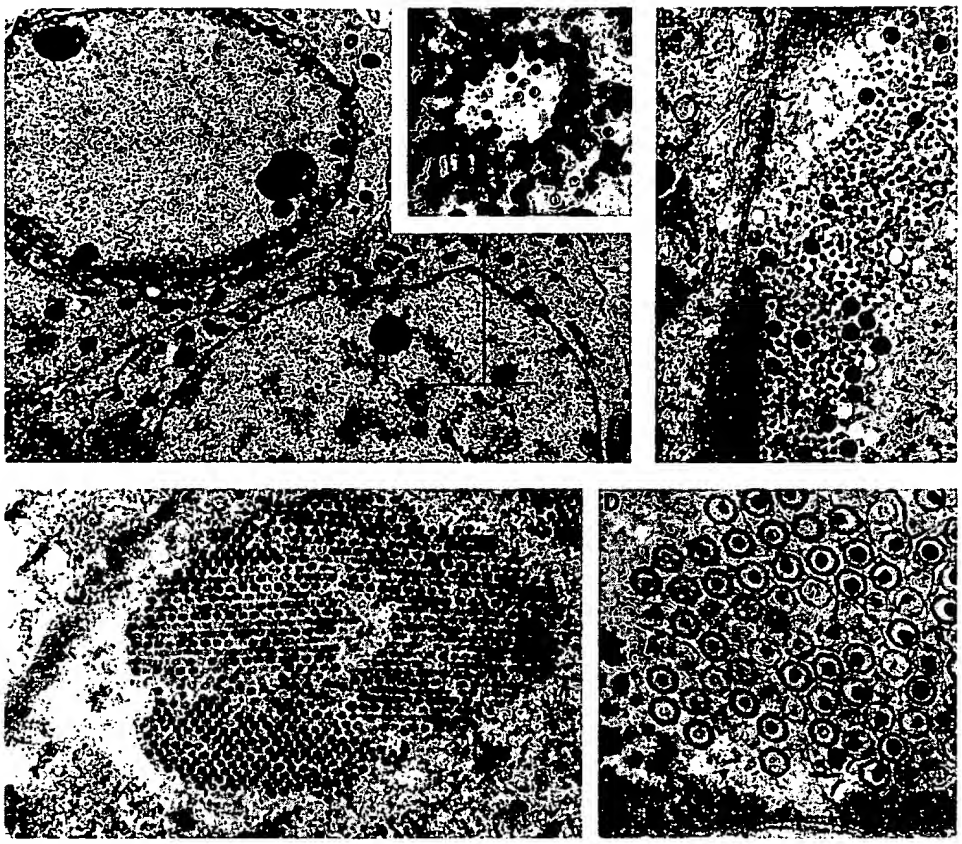

FIG. 4. Electron micrographs of the intracellular events in HSV-1 replication. **A:** Electron-opaque bodies (magnified in insert) showing sites of assembly of capsids. **B:** A region near the end of the nucleus showing accumulation of chromatin, small particles that appear to be capsid precursors and capsids. **C:** A paracrystalline array of capsids, both empty and containing DNA, frequently found in nuclei of infected cells. **D:** Capsids in nuclei of infected cells in various stages of packaging of viral DNA. Electron micrographs assembled from Roizman and Furlong (557) and Schwartz and Roizman (600,601), with permission.

volved in attachment was obtained in studies on the inhibition of attachment of HSV to BHK cells by the polycation neomycin (75). In this study, it was shown that the resistance of HSV-2 to inhibition of attachment by neomycin mapped to the gC-2 gene. It was later reported that deletion of the membrane anchor domain of gC decreased, but did not eliminate, the ability of purified virions to attach to nonpolarized cells in culture (245). When polarized epithelial cells were used in attachment assays, it was shown that gC is required for attachment of HSV virions to a receptor found on the apical surface of polarized cells, but not for attachment of the virus to the basal surfaces of the same cells (605). Polarized epithelial cells in culture therefore express more than one receptor for HSV, and those receptors are recognized by different viral proteins.

This attachment to different cell surface receptors mediated by different viral glycoproteins raises the possibility that nonpolarized cells may express more than one receptor on the same surface, and at least some of the myriad of apparently nonessential glycoproteins encoded by this virus may then be functionally redundant in infection of nonpolarized cells. In that case, removal of one glycoprotein at a time cannot completely eliminate attachment, since the viral protein required for attachment to another receptor would still be present. Viruses lacking gC, gG, and gE, or gE and gI, are able to attach to and infect nonpolarized cells (A. Sears and B. McGwire, unpublished data). Because gB is also a strong binder of heparan sulfate, it has been postulated (245) that gB is responsible for gC-independent attachment. However, this hypothesis depends upon heparan sulfate being the sole viral receptor, which no longer appears likely.

Spear et al. (206,622,747) have identified cell surface heparan sulfate as a major factor in binding of HSV to the cell surface; heparin is a potent inhibitor of HSV attachment, and removal of heparan sulfate from cells either enzymatically or by the use of mutant cell lines deficient in heparan sulfate synthesis reduces the levels of virus attachment to and infection of those cells by approximately 85%. However, in no case has removal of heparan sulfate or competition by heparin resulted in a complete loss of

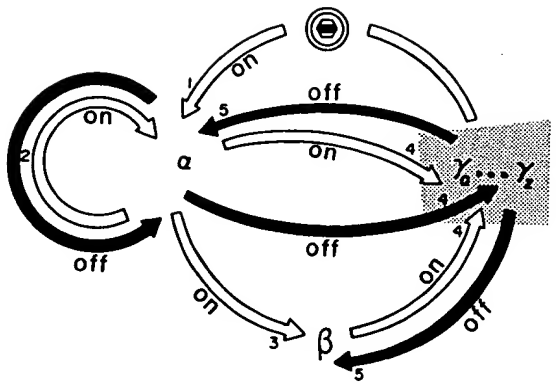

FIG. 5. Schematic representation of the regulation of HSV-1 gene expression. *Open* and *filled arrows* represent events in the reproductive cycle which turn gene expression "on" and "off," respectively. **1:** Turning on of α gene transcription by α-TIF, a γ protein packaged in the virion. **2:** Autoregulation of α gene expression. **3:** Turning on of β gene transcription. **4:** Turning on of γ gene transcription by α and β gene products through transactivation of γ genes, release of γ genes from repression, and replication of viral DNA. Note that γ genes differ with respect to the stringency of the requirement for DNA synthesis. The heterogeneity is shown as a continuum in which inhibitors of viral DNA synthesis are shown to have minimal effect on γ_a gene expression but totally preclude the expression of γ_z genes. **5:** Turn off of α and β gene expression by the products of γ genes late in infection.

attachment or infectivity. Two possible explanations for this are that heparan sulfate is one of multiple receptors, or that it acts as a cofactor, enhancing binding of gC or other glycoproteins to cell surface proteins.

Another factor that has been implicated in attachment of HSV is the basic fibroblast growth factor receptor (FGFR). Reports that the presence of this protein on the cell surface was required for viral attachment (301) could not be substantiated by other laboratories (431,445,621). However, inasmuch as there is more than one attachment pathway available to HSV in infection of nonpolarized cells, the possibility that FGFR is one of multiple receptors was not conclusively ruled out.

Recent experiments have identified the receptor for gC-dependent attachment of HSV-1 to the apical surface of polarized epithelial cells as the complement receptor CR1 (CD35; A. Sears, in preparation). This protein, closely related to the Epstein-Barr virus receptor CR2, has a wide tissue distribution *in vivo* (479). The role of heparan sulfate in the gC-CR1 interaction has yet to be determined.

Penetration into the Infected Cell

Attachment to the cell surface activates a process mediated by viral surface proteins which causes the fusion of the viral envelope and the cell plasma membrane. There is overwhelming acceptance of the hypothesis that productive infection results from the entry of virus mediated by fusion of the envelope and plasma membranes rather than that mediated by phagocytosis (440). There is also evidence that fusion of the envelope with membranes lining endocytic vesicles results in a nonproductive infection (70). The demonstration that virion envelope Fc receptors (i.e., glycoproteins gE and gI) (291) could be detected on cell surfaces following penetration in the absence of viral gene expression is consistent with this hypothesis (474).

Penetration may be a multistep event and involves more than one viral glycoprotein. In nonpolarized cells, the cumulative evidence indicates that (a) a ts mutant in HSV-1 gB attaches to but does not penetrate into cells (386), but infection does ensue, and progeny virus is made after chemically induced fusion of the envelope of the adsorbed virus to the plasma membrane (590,591); (b) HSV-1 gB^-, gD^- and gH^- recombinant viruses also attach to cell surfaces but do not penetrate (66,190,289); (c) cells expressing HSV-1 gD allow attachment and endocytosis of both HSV-1 and HSV-2, but fusion of the membranes and penetration do not ensue (70); and (d) viruses deleted in gL attach to but do not penetrate into cells; however, the gL^- virions also lack gH (575), so that the role of gL in penetration remains to be seen. In the case of polarized epithelial cells, gG, gE, and gI are required for a postattachment stage of entry, the nature of the step(s) requiring gG, gE, and gI remains to be determined (A. Sears, B. S. McGwire, and L. Tran, in preparation).

The role of gD in viral penetration deserves a further note. Clonal lines originally derived from BHK cells have been shown to vary with respect to susceptibility to infection by wild-type HSV-1. Mutant viruses capable of infecting the clonal lines have been isolated. A mutation in gD capable of conferring on recipient virus some but not all of the properties of the mutant virus mapped in the gD gene (572). In addition, cell lines expressing HSV-1 gD allow attachment and endocytosis of both HSV-1 and HSV-2, but fusion of the membranes and penetration do not ensue (70). It appears that the gD expressed in these cells is responsible at least in part for the observed resistance to infection, that viral mutants which overcome the resistance contain mutations in gD, and that transfer of the mutations in gD to a recipient virus overcomes in part the resistance to infection. The evidence favors additional mutations in the viral genome necessary to overcome resistance to infection completely and, by extension, argues that entry of virus into cells requires the participation of several gene products (56).

The transition from attached to penetrated virus (as measured by the loss of susceptibility to neutralization characteristic of virus still attached to the cell surface) is very rapid (265).

Release of Viral DNA

Upon entry into the cell, the capsids are transported to the nuclear pores (Figs. 3 and 7) (32,693). Release of viral

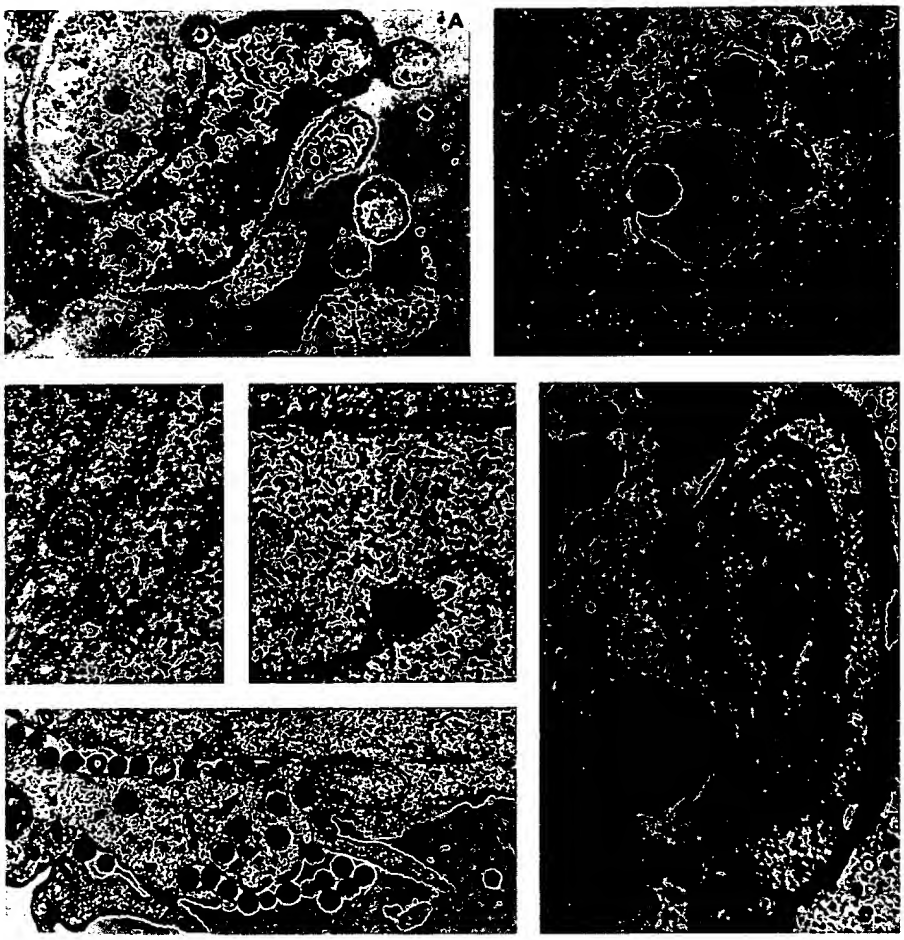

FIG. 6. Electron micrographs of the envelopment and egress of virus from infected cells. **A:** Envelopment of virus from a protrusion of the nucleus. Note that the nucleus contains marginated chromatin. The inner lamellae of the nuclear membrane contain electron-dense, slightly curved patches representing regions of the membrane at which envelopment takes place. Note the spikes projecting from the surface of the membrane of the capsid being enveloped. **B:** An enveloped capsid and numerous unenveloped capsids found late in infection in the cytoplasm of infected cells. Some of the capsids appear to be in the process of being either enveloped or deenveloped. **C:** Micrograph showing an enveloped capsid in the space between the inner and outer lamellae of the nuclear membrane connecting with the cisternae of the endoplasmic reticulum. **D:** An unenveloped capsid in the nucleus and an enveloped particle bulging in the cisternae of the endoplasmic reticulum. **E:** Cytoplasmic enveloped particles enclosed in vesicles or cisternae of the endoplasmic reticulum. **F:** Modified nuclear membranes folded upon themselves, frequently seen in cells late in infection. The structures formed by such membranes have been designated as "reduplicated membranes." Electron micrographs assembled from Roizman and Furlong (557) and J. Schwartz and B. Roizman (unpublished micrographs).

DNA into the nucleoplasm requires a viral function; thus, capsids of the ts mutant HSV-1(HFEM)*ts*B7 accumulate at nuclear pores and release viral DNA only after a shift down from nonpermissive to permissive temperature (32). Empty capsids are readily found at nuclear pores early in infection with wild-type viruses. The cellular cytoskeleton probably mediates the transport of herpesvirus capsids to the nuclear pores (124,331). Parental viral DNA accumulates in the nucleus.

Virion Components Required for Replication in Permissive Cells

Transfection of "permissive" cells with intact, deproteinated viral DNA yields infectious viral progeny (225,351,616). However, the specific activity of viral DNA is many orders of magnitude lower than that of virions, and the duration of the reproductive cycle is longer. Moreover, there is no certainty that the sequence of events in trans-

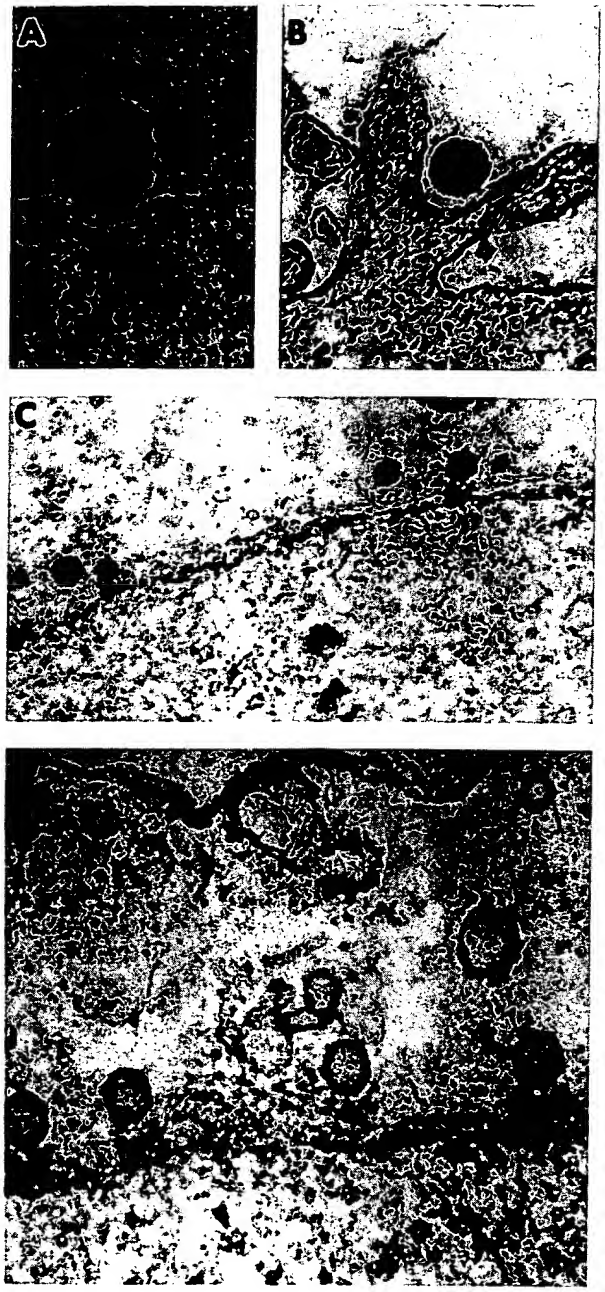

FIG. 7. Attachment and penetration of HSV-1 to cells in culture. **A,B:** Virions attached to plasma membrane. **C:** Capsids with DNA at nuclear pores in cells infected with HSV-1(HFEM)*ts*B7 maintained at the nonpermissive temperature (32). **D:** Empty capsids accumulating in cells late in infection with mutant HSV1(50B) late in infection (693). In cells infected with this mutant, virtually every pore contains a juxtaposed empty capsid.

fected cells resembles the viral reproductive cycle occurring in cells infected with competent virions.

The components of the virion other than its DNA appear to have several functions. The envelope obviously enables entry of the virus into cells, and the capsid acts a vector for the viral DNA. Several tegument proteins have been shown to have important functions for the initiation of viral replication, and surprises are yet to come. Among these are (i) VHS (U_L41), which is involved in the early shut off of host macromolecular synthesis (341,342,458,457,530, 554,595, 675–677); (ii) a protein designated by the Spear and Roizman (643) nomenclature as VP16 acts in *trans* to induce α genes, the first set of genes to be expressed (33,76, 481,498) (since the induction of α genes is a nuclear event, it is evident that at least some tegument components make their way into the nucleus); (iii) two proteins (U_L46, U_L47), which appear to modulate the function of VP16 (400a); (iv) a protein encoded by U_S11, which binds the 60S ribosomal subunit and whose function is not known (573); (v) a protein kinase (U_L13) whose function in newly infected cells is not known (520,521); and (vi) proteins which may facilitate attachment to the nuclear pore and the release of viral DNA (32).

Preston and Notarianni (506) reported adenosine diphosphate (ADP) ribosylation of VP23, a capsid protein (213,643). It has previously been reported that phosphate cycles on and off VP23, suggesting that a kinase associated with virions (although not necessarily viral in origin) phosphorylates and dephosphorylates VP23 and substrate proteins (360).

It should be noted that 38 of the 75 open reading frames encoding unique proteins are dispensable at least in some cell lines and that a large proportion of the dispensable open reading frames are structural proteins, particularly of the tegument and envelope. The functions of the dispensable proteins are particularly important inasmuch as they may eventually identify the cellular functions required for viral replication in specific tissues or cell types.

Viral Genes: Pattern of Expression and Characterization of Their Products

Timing and Requirements for Gene Product Synthesis

The transcription of viral DNA takes place in the nucleus. As would be expected, all viral proteins are synthesized in the cytoplasm. The number of abundant, i.e., readily detectable, polypeptides specified by HSV does not exceed 50 (116,262,442). McGeoch et al. (402,405) predicted 56 open reading frames in U_L, 12 in U_S, and one each in the repeats flanking the L and S components for a total of 72. However, as discussed later in this text, the definition of open reading frames in the viral genome was somewhat conservative, and the actual number is higher. Since the HSV-1 sequence was published, ten additional transcribed open reading frames have been reported. These are $\gamma_1$34.5, ORF, and ORF P, which map in the repeats flanking the L component and therefore present in two copies, $U_L26.5$, which encodes a capsid scaffolding protein, and the substrate of the protease, $U_L49.5$, $U_L20.5$, $U_S1.5$, and $U_S8.5$ (5,28,97,211,346, 370,371, P. L. Ward, K. Carter, and B. Roizman, in preparation.). The functions of ORF P, $U_L49.5$, and $U_S8.5$ are not known.

In cells productively infected with HSV, the regulation of viral gene expression schematically represented in Fig. 5 has three features: (i) HSV proteins form several groups whose synthesis is coordinately regulated in that they have similar requirements for and kinetics of synthesis, (ii) the absolute rate of synthesis and ultimate abundance of each protein may vary, and (iii) the protein groups are sequentially ordered in a cascade fashion (183,260–262,329,483).

The α genes are the first to be expressed. There are five α proteins, i.e., ICP0, ICP4, ICP22, ICP27, and ICP47 (Table 1). The α genes were initially defined as those that are expressed in the absence of viral protein synthesis. The α genes may be defined more precisely by the presence of the sequence 5′ NC GyATGnTAATGArATTCyTTGnGGG 3′ in one to several copies within several hundred base pairs upstream of the cap site (382).

The synthesis of α polypeptides reaches peak rates at approximately 2 to 4 hr postinfection, but α proteins continue to accumulate until late in infection at nonuniform rates (4,261). As discussed below, all α proteins, with the exception of α47, have been shown to have regulatory functions. and functional α proteins are required for the synthesis of subsequent polypeptide groups.

The ß genes are expressed at very low levels in the absence of competent α proteins, and their expression is enhanced rather than reduced in the presence of inhibitory concentrations of drugs that block viral DNA synthesis or in cells infected with tight DNA-ts mutants in ß genes. The $ß_1$ and $ß_2$ groups of polypeptides reach peak rates of synthesis at about 5 to 7 hr postinfection (260,261). The $ß_1$ proteins, exemplified by polypeptides ICP6 (the large component of the viral ribonucleotide reductase) (270) and ICP8 (the major DNA binding protein) (108) appear very early after infection and in the past have been mistaken for α proteins (102). They are differentiated from the latter by their requirement for functional α4 protein for their synthesis (260,261). The $ß_2$ polypeptides include the viral thymidine kinase (TK) and DNA polymerase. The appearance of ß proteins signals the onset of viral DNA synthesis, and most viral proteins involved in viral nucleic acid metabolism appear to be in the ß group.

The γ genes have been lumped for convenience into two groups, $γ_1$ and $γ_2$, although in reality they form a continuum differing in their timing and dependence on viral DNA synthesis for expression (108,113,300,625,707). The prototype $γ_1$ gene (e.g., the genes specifying glycoproteins B and D and γ34.5) is expressed relatively early in infection and is only minimally affected by inhibitors of DNA synthesis. The relatively abundant major capsid protein ICP5 ($γ_1$) is made both early and late in infection. In contrast, prototypic $γ_2$ proteins [e.g., gC (U_L44) and U_S11] are expressed late in infection and are not expressed in the presence of effective concentrations of inhibitors of viral DNA synthesis.

The $γ_1$ genes have also been designated as ßγ or leaky γ genes (114,253). The differentiation of ß genes into $ß_1$ and $ß_2$ and the variability in the requirements for the expression of genes are the major reasons for the designation of HSV genes as α, ß, and γ rather than immediate-early, early, and late (261).

Functi nal Organization of HSV Genomes

The sources of the data for the functional organization of the HSV-1 genome shown in Fig. 8 are useful to present for both historical and heuristic reasons. Globally, the key sources were the transcriptional maps painstakingly collected and defined by E. K. Wagner et al. (11,12,113,114, 151,200,230,253–255,707,709). These maps served as the basis for the interpretation of the nucleotide sequence data generated by McGeoch et al. (402,405), although in some instances transcriptional analyses and even translational analyses were ignored in favor of nucleotide sequences denoting putative transcriptional initiation sites or terminations. Identification of the proteins specified by the individual open reading frames is based on several sources. The framework and much of the initial mapping of the HSV genome is based on analyses of proteins and DNA sequence arrangements of HSV-1 × HSV-2 recombinants (390,441, 442,582) supplemented by (i) rescue of mutants by transfection of cells with intact mutant viral DNA and DNA fragments generated by restriction endonuclease digestion of wild-type genomes (319,320,444,476), (ii) transfer of a dominant or assayable marker from one genome to another with restriction endonuclease fragments (320,327,498, 499,582), (iii) expression of the gene product from purified messenger RNA (mRNA) or from a DNA fragment in a suitable system (108,247,355,383,498), and (iv) insertion in frame with the putative open reading frame of a "tag" consisting of a nucleotide sequence encoding a non-HSV epitope for a known monoclonal antibody (346,370, 371). The products of a large number of putative open reading frames have not been identified. The sequence-dependent, in contrast to transcription- or function-dependent, identification of open reading frames is conservative and does not take into account proven exceptions (e.g., the arbitrary rules would have excluded α0 as an open reading frame had its product not been known). Nevertheless, the overall organization of the genome is becoming apparent and can be summarized as follows:

1. The α genes map near the termini of the L and S components (11,299,300,383,441,448,508,714,715). The α0 and α4 genes map within the inverted repeats of the L and S components, respectively,and are therefore each present in two copies per wild-type genome. However, a single copy of each is sufficient inasmuch as I358, a HSV-1 mutant lacking most of the internal inverted repeat sequences, is viable (490). In the circular arrangement of viral DNA, the α genes form two clusters. The first consists of α genes 0, 4, and 22, whereas the second consists of α genes 47, 4, and 0. A key feature of these two clusters is that each con-

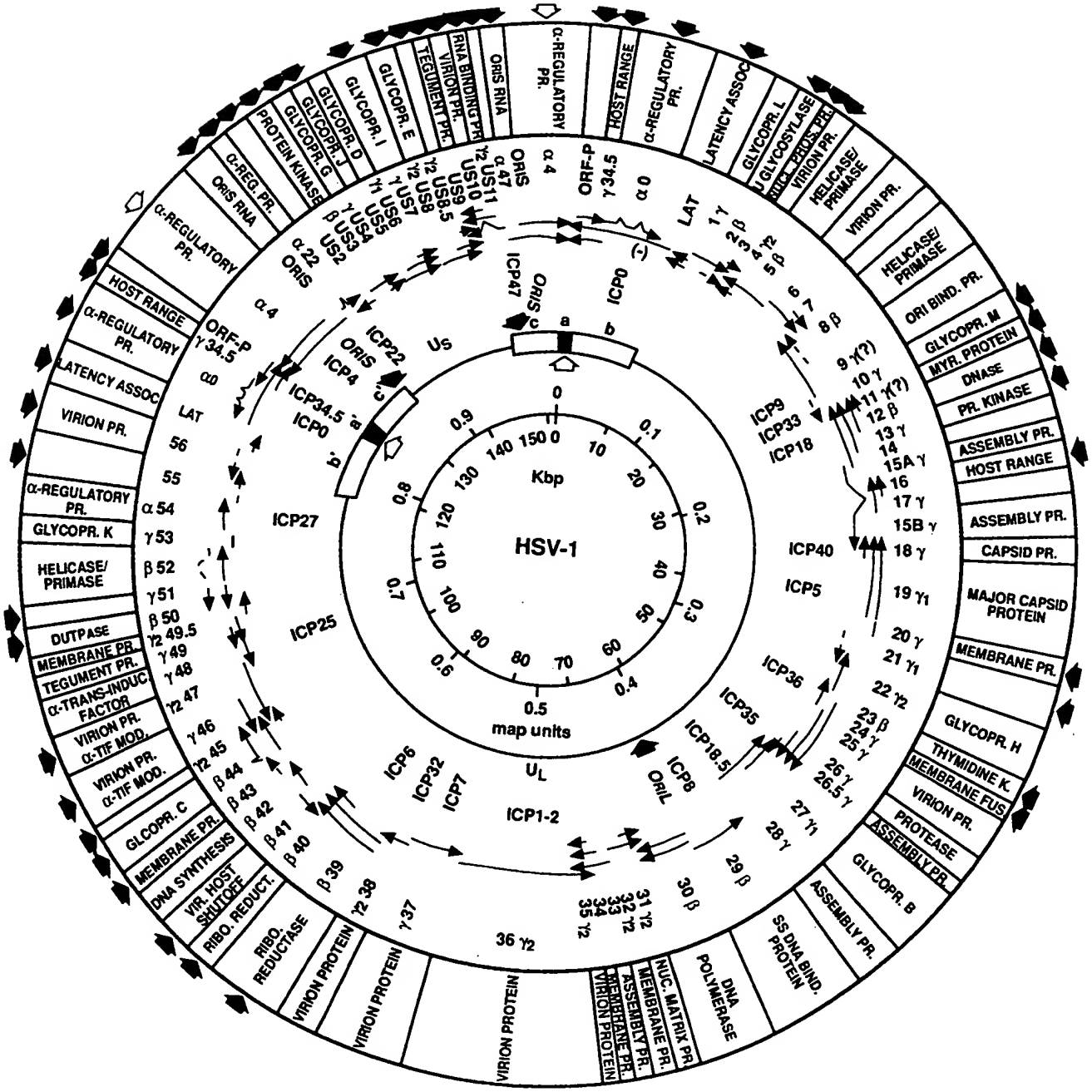

FIG. 8. Functional organization of the HSV-1 genome. The circles are described from inside out. **Circle 1:** Map units and kilobase pairs. **Circle 2:** Sequence arrangement of HSV genome. The letters *ab*, U_L, and *b´a´* identify the long (L) component consisting of the unique sequences U_L flanked by the inverted repeats. The letters *a´c´*, U_S, and *ca* identify the corresponding sequences of the short (S) component. The *open arrow* shows the sites of cleavage of concatemeric or circular DNA to yield linear DNA. Since the L and S components can invert relative to each other, the arrangement shown is that which would yield the "prototype arrangement" if linearization were to occur by cleavage of the DNA between map units 0 and 100. The *filled arrows* represent the three origins of viral DNA synthesis, one in the middle of U_L (OriL) and two (both designated OriS) within the inverted repeats flanking the S component. **Circle 3:** The transcriptional map of the HSV-1 genome. The map serves the purpose of identifying the direction of transcription, the approximate initiation and termination sites, and the families of 3´ coterminal transcripts. *Dashed lines* identify transcripts mapped imprecisely. The designation between the second and third ring identify proteins encoded by the transcripts according to their ICP number. The designations outside this ring identify the open reading frame (those mapping in U_S have the prefix US) number and the kinetic class (α, ß, or γ) to which they belong. **Circle 4:** The known functions of the proteins specified by the open reading frames. The *filled arrows* identify open reading frames which can be deleted without affecting the ability of the virus to multiply in cells in culture. The *open arrows* identify the two copies of the α4 gene; only one copy of this gene can be deleted without affecting the capacity of the virus to multiply. The data for the circles 3 and 4 are derived from refs. 11, 28, 53, 81, 97, 113, 125, 150, 168, 200, 201, 230, 255, 280, 346, 371, 402, 402, 411, 416, 448, 481, 484, 523, 537, 540, 614, 707, and 716.

tains an origin of DNA synthesis (Ori_s) sandwiched between α4 and α22 or between α4 and α47. Notwithstanding the clustering, each α gene has its own promoter-regulatory region and transcription initiation and termination sites (381–383).

2. With few exceptions, ß and γ genes are scattered in the unique sequences of both the L and S components. The exceptions are the $\gamma_1$34.5 and ORF P genes located in the reiterated sequences flanking the L component between the terminal *a* sequence and the α0 gene (6,97,346). At present only two functional gene clusters are strikingly apparent, but their significance is uncertain. The ß genes specifying the DNA polymerase and the single-stranded DNA-binding protein flank the L component origin of DNA synthesis (Ori_l), and the γ genes specifying membrane glycoproteins D, E, G, and I and the presumed glycoprotein J map next to each other within the unique sequences of the S component (6,191,354, 535,561,582,640,716). Although there are several instances of apparent sharing of 5′ or 3′ gene domains (151,537,714), there is altogether little gene overlap (ORF P and γ_1 34.5) and only a few known instances of RNA splicing (e.g., α22, α47, α0, and U_L15) (707) relative to the frequency with which these events have been observed to occur in adenovirus and papovavirus genomes. In several instances, replacement of the genomic DNA sequences of spliced genes with the corresponding complementary DNA (cDNA) had no effect on viral replication in cells in culture (21).

A more detailed listing of the gene products is presented in Table 1. Each viral protein is designated by one of three criteria, i.e., by function, if it is precisely defined (e.g., tk, DNA polymerase, and so forth), by its first published designation, or by its open reading frame.

Synthesis and Processing of Viral Proteins

Viral proteins appear to be made on both free and bound polyribosomes. Most of the proteins examined to date appear to be processed extensively after synthesis (4,43,44,59, 74,167,213,263,384,483,506,521–523,639–641). Processing includes cleavage, phosphorylation, sulfation, glycosylation, myristylation, ADP-ribosylation, and nucleotidylylation. In some instances, the modifications in protein structure accompany the translocation of proteins across membranes (393). Current information concerning processing of proteins and the relationship of processing to function is detailed in the section on general properties and functions of viral proteins and in the section on viral glycoproteins.

With the exception of some glycoproteins, the extent to which processing is a requirement of virus growth rather than the consequence of an encounter between cellular or viral enzymes and molecules resembling natural substrates remains uncertain. N-linked glycosylation of gD is not necessary for its function (637). However, at least some glycosylation within the infected cells is necessary to produce infectious progeny (74,639).

HSV Protease

Gibson and Roizman (213,215) noted that protein VP22a present in empty B capsids was replaced in DNA-containing C capsids by a faster migrating protein with similar characteristics, designated as VP22. VP22a proteins were identified as members of a family of proteins designated as ICP35a-f (59,60). VP22a, corresponding to ICP35e,f are derived from ICP35c,d and form the toroidal structure which functions as a scaffolding for DNA packaging into capsids (451). As predicted from early studies (213), it is absent from DNA-containing capsids. McGeoch et al. (403) assigned ICP35 to the open reading frame U_L26 on the basis of the observation that a temperature-sensitive mutation affecting the processing of ICP35 and accumulation of mature capsids mapped in the U_L26 open reading frame (507). Numerous studies (144,368–371) indicate the following:

1. The domain of U_L26 contains two independently transcribed open reading frames, U_L26 and U_L26.5. U_L26 encodes a protein of 635 amino acids. The U_L26.5 promoter maps within the coding domain of U_L26. U_L26.5 encodes 329 amino acids; the methionine initiator codon of U_L26.5 corresponds to the methionine 306 of U_L26. Although they are transcribed separately and although the abundance of the U_L26.5 product is higher than that of U_L26, the product of U_L26.5 has the same amino acid sequence as the carboxy-terminal amino acids of U_L26.
2. U_L26 is a serine protease (368,369,371). U_L26 cleaves itself between Ala^{610} and Ser^{611} and between Ala^{247} and Ser^{248} (144,368,371). The amino-terminal 247-amino acid polypeptide of U_L26 retains its catalytic activity (368). The carboxy-terminal product of the cleavage of the product of the U_L26 gene between Ala^{247} and Ser^{248} exists in two states, i.e., with and without the 25 carboxy-terminal amino acids; these proteins correspond to the ICP35a,b of Braun et al. (58). The protease also cleaves the terminal 25 amino acids at the corresponding Ala-Ser site off the U_L26.5 product ICP35c,d to form ICP35e,f. Mutagenesis studies have identified two histidines and two glutamic acid residues essential for proteolytic activity, but a conserved cysteine was dispensable in the 247-amino acid polypeptide (368,369).
3. The unprocessed product of the U_L26.5 gene and ICP35e,f form double bands. The modification of the protein which causes double band formation maps in the domain of the U_L26.5 gene between codons 307 and 417 (368).
4. At this time, no other substrates have been identified for the U_L26 protease.

Protein Kinases

Herpes simplex virus appears to specify at least three protein kinases. One protein kinase activity has been associated with the large subunit of the ribonucleotide reductase (ICP6) of HSV-2 but not of HSV-1 (100). In contrast to the carboxy-terminal domain, the amino-terminal 300+-amino acid stretch of the HSV-2 polypeptide shares little homology with the corresponding domain of the HSV-1 homolog (455). Whereas the ribonucleotide reductase activity is associated with the conserved carboxy-terminal domain, the protein kinase activity maps near the amino terminus (9,109). The large subunit of ribonucleotide reductase appears to be a multifunctional protein. The observation that HSV-2 but not HSV-1 evolved this activity of the ribonucleotide reductase is surprising. The substrate specificity of this protein kinase activity is not known.

McGeoch and Davison (404) predicted that U_S3 encodes a protein kinase on the basis of its sequence, a conclusion verified by studies with antibody to the protein (193) and with deletion mutants in the gene (519). The major substrate of this enzyme is an intrinsic membrane protein exposed on the surface of infected cells and encoded by U_L34. The U_L34 protein contains the amino acid motif recognized by the U_S3 protein kinase; substitution of the serine or threonine residues within this motif with alanine resulted in a loss of phosphorylation, but the virus grew poorly, and revertants were readily detected. It is noteworthy that four prominent phosphoproteins unrelated to the U_L34 protein appear in lysates of cells infected with the U_S3^- mutant or with mutants in the amino acid motif recognized in U_L34 by the U_S3 kinase. The anti-U_L34 serum coprecipitates the four phosphoproteins along with the U_L34 protein. It would appear that the four phosphoproteins compensate in some fashion for the absence of phosphorylation by U_S3 kinase. Undoubtedly, the U_S3 kinase phosphorylates other as yet unidentified proteins (522,523).

Smith and Smith (636) and Chee et al. (88) reported that the sequence of the U_L13 open reading frame contains the signature common to other protein kinases and is shared among α, ß, and γ herpesviruses. Cunningham et al. (121) reported on the properties of a new kinase very similar to the protein kinase activity demonstrated in tegument-capsid structures described by LeMaster and Roizman (360) and ascribed the new kinase to the product of the U_L13 gene. Studies of U_L13^- and $U_L13^-U_S3^-$ mutants led to the conclusion that the U_L13 kinase affects the phosphorylation and processing of the gene products of α22 and U_L47 and the accumulation of the α0, U_L26 and $U_L26.5$ (protease and its substrate), and U_S11 gene products (520,521).

Although the U_L13 kinase is associated with structural proteins, the enzyme brought into the infected cell by the virion does not phosphorylate the newly synthesized ICP22 (520,521). The phenotype of the U_L13^- virus is similar to that described for the $\alpha22^-$ mutant by Sears et al. (603).

A large number of other proteins (e.g., ICP4 and gE; see Table 1 for a more detailed list) are phosphorylated in the course of the reproductive cycle. The kinases responsible for the phosphorylation and the role of phosphorylation in the functions of these gene products have not been elucidated.

ADP-Ribosylation

Preston and Notarianni (506) reported that ICP4 and VP23 are poly(ADP-ribosyl)ated in isolated nuclei, a significant finding that nevertheless left unanswered the question whether this reaction actually takes place in the infected cell. Blaho et al. (43) reported that antibody specific for poly(ADP-ribose) reacts with ICP4 extracted from cells late in infection, in effect answering the question in the affirmative. However, the poly(ADP-ribose) added to ICP4 was digested by poly(ADP-ribose) glycohydrolase, but only after denaturation of the protein. In contrast, poly(ADP-ribose) added to ICP4 in isolated nuclei was readily removed from the native protein by the glycohydrolase. The results indicate that ICP4 is poly(ADP-ribosyl)ated and suggest that, in the isolated nuclei, the poly(ADP-ribose) is added either by elongation of existing chains or to novel sites.

Nucleotidylylation of Viral Proteins

An initial report by Blaho and Roizman (46) showed that ICP4 is both guanylylated and adenylylated. The label transferred by α^{32}P-adenosine triphosphate (ATP) or α^{32}P-guanosine triphosphate is associated with the slowly migrating forms of the protein. Conclusive evidence for the nucleotidylylation emerged from transfer of a ^{3}H-labeled purine ring from ATP to ICP4. More recent studies revealed that an as yet unidentified late viral gene product was involved in the reaction and that the number of nucleotidylylated proteins is higher (43,44). The first to be identified in addition to ICP4 were ICP0, ICP22, and ICP27. ICP22 and ICP27 share the amino acid sequence Arg/ProArgAlaPro/SerArg which is also found in ICP4, ICP0, and the products of the HSV-1 genes U_L21, U_L31, U_L47, and U_L49. ICP0, ICP4, ICP22, and ICP27 are regulatory proteins; U_L21 is dispensable for growth in cultured cells (24); U_L31 cofractionates with the nuclear matrix (85); and U_L47 may interact stoichiometrically with αTIF (or VP16, the product of the U_L48 gene) (425). U_L49 is a virion protein which is labeled in cells with [^{32}P]orthophosphate and also is (ADP-ribosyl)ated. The genes encoding the additional nucleotidylylated proteins were identified initially by analyses of intertypic recombinant viruses and subsequently by analyses of the products encoded by the mapped genome domains and were shown to correspond to U_L21, U_L31, U_L47, and U_L49 proteins (44). Preliminary evidence indicates that casein kinase II nucleotidylylates at least ICP22 and that an additional late viral protein is required for nu-

cleotidylylation of most of the remaining seven proteins (45,432). The significance of the nucleotidylylation is not known. In fact, there is no evidence that the identified "consensus" sequence is nucleotidylylated.

Modification of Membrane-Associated Proteins

Of the 11 predicted glycoproteins, at least ten have been studied in sufficient detail to demonstrate the presence of oligosaccharide chains. The 11th, gJ, remains elusive. In addition, it has been reported recently that the products of the U_L47 gene, VP13-14, contain O-linked polysaccharide chains and are phosphorylated (425).

At least one protein, the product of the U_L11 gene, has been shown to be myristylated (384). Deletion mutants in the U_L11 gene show an impairment in egress from infected cells (20).

Application of Genetic Techniques to the Identification of Gene Product Function: Genes Essential and Dispensable for Growth in Cell Cultures

Key to the identification of viral functions and mapping of viral genes encoding these functions are temperature sensitive (ts) and null mutants. Earlier studies identified approximately 30 complementation groups (723)—an extraordinary accomplishment in itself given the difficulties inherent in the selection and testing of the numerous mutants produced by many laboratories. The ts mutants have been enormously helpful in mapping genes. Nevertheless, this approach to identification and mapping of viral functions suffers from several problems, i.e., (i) the phenotypes of viruses containing extensive mutations in some nonessential genes cannot be readily differentiated from that of the wild-type parent, (ii) conditional lethal (e.g., ts) mutants produced by general mutagenesis of the viral genome may contain a large number of silent nonlethal mutations in both essential and nonessential genes, (iii) the phenotypes of mutations introduced into domains shared by more than one gene cannot be readily attributed to the malfunction of a specific gene product, and (iv) while the usefulness of ts mutants is in part dependent on their efficiency of plating at permissive and nonpermissive temperatures, tight mutants with high permissive/nonpermissive ratios may well contain more than one point mutation. Although the presence of multiple mutations in a single gene should not affect the mapping or identification of the gene function, it does present a problem in mapping the functional domains of the gene.

An alternative to the random or fragment-specific substitution of bases in DNA is site-specific deletion of the viral genome. A protocol for site-specific insertion/deletion of viral genes was first reported by Post and Roizman (499). It was based on selection of recombinants generated by double recombination through homologous flanking sequences between an intact viral DNA molecule and a DNA fragment containing an insertion or deletion and a selectable marker. The selectable marker used in these studies was the viral TK gene (*tk*) because (i) it can be deleted from the HSV genome without affecting the growth of virus in cell culture, (ii) a plasmid-borne *tk* gene can be altered so that it cannot recombine by double crossover to repair the deletion in the genomic *tk* gene, (iii) viruses carrying a functional *tk* gene can be selected against by plating viral progeny in the presence of nucleoside analogs phosphorylated by the viral TK (e.g., AraT), and (iv) viruses expressing the *tk* gene can be selected for by plating the virus in TK^- cells in medium containing methotrexate or aminopterin, which block the conversion of thymidine monophosphate (TMP) from deoxyuridine monophosphate (dUMP) by thymidylate synthetase and precludes the *de novo* pathway of TMP synthesis. This procedure permits the selection of viable mutants with deletions or insertions in genes which appear to be nonessential for growth in cells in culture. Other investigators adapted the double crossover protocol for selection of mutants with deletions in essential genes (66,137,363). In this protocol, the gene to be deleted was transfected into and expressed in cells in culture, and the host cell line, i.e., the cells expressing the gene, was then transfected with intact viral DNA and the mutated DNA fragment. The progeny of transfection were screened for deletion mutants that multiplied only in the vector cell line.

A still different protocol for insertional mutagenesis is based on the use of transposons (e.g., miniMu phage, Tn5) (285,558,718). Its principles were described first by Jenkins et al. (285), taking advantage of the random insertion of the DNA of phage miniMu into target plasmid DNAs. A miniMu phage was constructed containing a modified HSV-1 *tk* gene. Transposition of this miniMu into an HSV fragment is random and limited to one insert per plasmid copy. Transfection of intact tk^- viral DNA with an HSV DNA fragment containing random insertions of the modified miniMu would result in recombinants in which the miniMu, which is randomly inserted into the viral DNA fragment, would become recombined at the identical position in the viral genome. However, only the HSV genomes containing the miniMu sequences at nonessential sites multiplied in cells in culture.

Although the transposons have a technical advantage of reduced labor to produce the insertion mutants, they suffer from the fact that the target gene is not actually deleted and the site of insertion must be ascertained precisely since truncated genes may still yield a functional product.

The genes known to be dispensable for growth in cells in culture are listed in Table 1. It should be stressed that most of the dispensable genes are required for replication in experimental animal systems and that in no instance has

a virus lacking a dispensable gene been isolated from human lesions (although viruses that fail to react with a specific monoclonal antibody are readily isolated).

Genes dispensable for viral replication in cells in culture fall into several groups whose products are involved in entry of HSV into cells, regulation of gene expression, posttranslational modification of proteins, exocytosis, inhibition of host response to infection, and spread of virus from cell to cell.

In a special category are deletion mutants whose ability to multiply is cell species-dependent. One example of such mutants is the $\alpha 22^-$ virus, which grows well in Vero and HEp-2 cell lines but not in human fibroblast strains or in rodent cell lines (603). In the nonpermissive cells, the virus fails to express γ_2 genes efficiently. Another example of a cell-specific gene is $\gamma_1 34.5$, which enables HSV to multiply in human cells but is dispensable in Vero, baby hamster kidney cells, and so forth. In the absence of the gene, there is total shut off of protein synthesis before significant amounts of virus are synthesized (91,93,94).

It could be predicted that viral genes which specify products whose functions are identical and interchangeable with those of cellular genes would be dispensable, at least in cells which express these functions. In this category are the *tk* gene, the genes specifying ribonucleotide reductase, and so forth. The observation that some virion proteins are dispensable for infection and replication of virus at least in cell culture was very puzzling for many years.

Although we cannot exclude the possibility that cells express proteins with similar functions which complement the deletion mutants, a more likely scenario is that cells in culture express many more genes than cells *in situ* in animal organs. The exceptions to date are the polarized epithelial cells and the neuroblastoma cells in which the gene products are sorted differently or which differ from other cells in culture with respect to the nature of the genes that are expressed. Herpes simplex virus may carry a set of genes which enables the virus to enter (e.g., the dispensable glycoproteins), multiply (e.g., $\gamma_1 34.5$), or egress (e.g., $U_L 20$) from a wide variety of human cells. Since these genes are not required for replication in all cells, there exists the formal possibility that functional analogs of the viral gene are encoded and may be expressed by cells. The obvious examples are the *tk*, ribonucleotide reductase, and so forth. The less obvious homologs are the cellular protein kinases which substitute for some of the viral enzymes, the homolog of $U_L 20$ which enables virus to egress from a variety of cell lines other than Vero cells, and so forth. The complexity involved in defining the function of 38 genes whose products are dispensable for viral replication in at least some cells in culture is significantly offset by the fact that these genes are excellent probes for analyses of cellular functions which at least in some instances complement the missing viral function.

Synthesis of Viral DNA

Temporal Pattern of Synthesis

A characteristic of herpesviruses not shared by other animal nuclear DNA viruses is that they specify a large number of enzymes involved in DNA synthesis. Although the sequence of events in viral DNA replication is roughly known, many details are still lacking. In HSV-infected cells, viral DNA synthesis is detected at about 3 hr postinfection and continues for at least another 12 hr (272,553,554,562). The DNA is made in the nucleus (Fig. 9). Earlier studies relied on incorporation of labeled thymidine into viral DNA—a procedure that yielded biased results inasmuch as the deoxynucleotide triphosphate pool increases and becomes saturated early in infection. Hence, the rate of viral DNA synthesis, as determined by the use of labeled deoxynucleosides, appears to be highest relatively early in infection. Analyses of viral DNA synthesis by hybridization with specific probes suggest that the bulk of viral DNA is made relatively late in infection (272).

Structure of Replicating DNA

At least in HSV-1-infected cells, only a small portion of total input (parental) viral DNA is replicated (278). The DNA labeled during a pulse lacks free ends, i.e., it consists of circles or head-to-tail concatemers (277,278). Labeled precursors become incorporated into molecules banding at a higher density which sediment at a faster rate than intact double-stranded DNA. In alkaline sucrose density gradients, the bulk of the labeled DNA bands at a position expected for small single-stranded fragments. Early after the onset of viral DNA synthesis, parental DNA, circles, and linear branched forms can be found in the DNA banding at the density of viral DNA. These are replaced late in the reproductive cycle by large, rapidly sedimenting bodies of tangled DNA. Available evidence suggests that, at least late in infection, herpesvirus DNAs replicate by a rolling circle mechanism (37,277). Attempts to find "theta" forms of replicating DNA early in infection have not been successful.

Origins of DNA Replication

The origins of DNA replication in the HSV genome were initially deduced from the structures of defective genomes (196,599) and have more recently been operationally defined as those sequences which must be present in a fragment of HSV DNA in order for it to be amplified in permissive cells transfected with the fragment and either transfected or infected with helper virus (437,704). By this definition, HSV-1 and presumably HSV-2 each contain three origins of DNA replication. Two of the origins map in the *c* reiterated sequence of the S component between

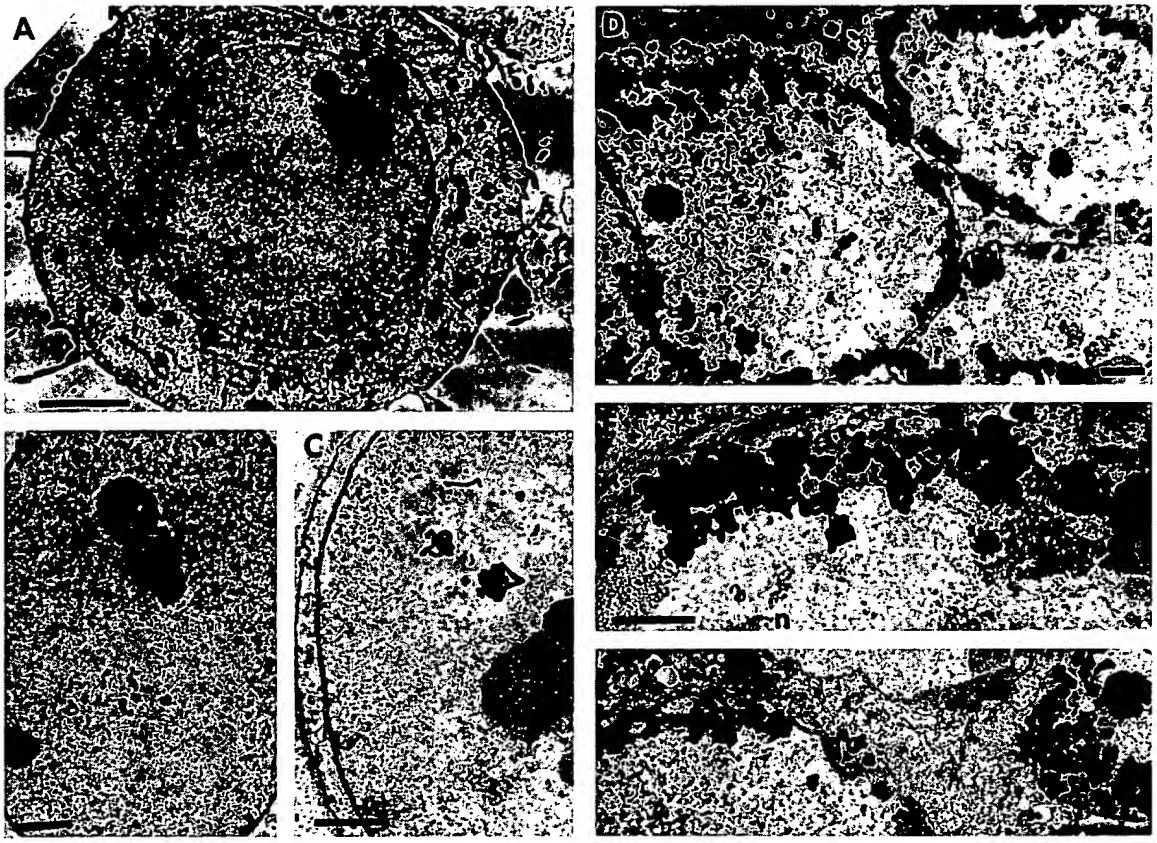

FIG. 9. Electron photomicrographs of thin-section autoradiography of HEp-2 cells infected with HSV. **A:** A 4-hr-infected cell pulse labeled for 15 minutes with ^{3}H-methyl thymidine prior to fixation. **B,C:** Enlargements of nuclei prepared as in part A. **D:** Portions of three nuclei of 18-hr-infected cells labeled with ^{3}H-methyl thymidine prior to infection. Unlabeled thymidine was present in the medium during and after infection. **E,F:** Electron micrographs of nuclei taken at higher magnifications. Note the aggregation of chromatin at the nuclear membrane. One of the cells in parts D and F did not synthesize DNA during the short labeling pulse. *n*, nucleus; *c*, cytoplasm; *v*, aggregation of virus-specific, electron-opaque material. From Roizman and Furlong (557) and J. Schwartz and B. Roizman (unpublished micrographs).

the promoters of α4 and α22 (ori_s1) or α4 and α47 (ori_s2) (29,131,437,665,666), whereas a third (ori_L) maps in the middle of the L component sandwiched between the promoters of the ß genes specifying the major DNA-binding protein (ICP8) and the DNA polymerase (373,725).

The L component origin consists of an A+T-rich sequence of 144 bp, forming a perfect palindrome (321,374, 527,725). Because of its extensive dyad symmetry, it tends to be unstable in DNA fragments cloned in *Escherichia coli*. The S component origin is smaller and contains a much shorter A+T-rich palindrome which is related to but lacks the complete dyad symmetry of ori_L. It has been suggested that the structure of ori_L enables bidirectional synthesis, whereas DNA synthesis initiated in ori_s would be unidirectional (725). The existence or necessity for bidirectional synthesis of DNA remains to be established. Earlier studies have shown that ori_L (494), and at least one ori_s (377) but not both (633) are dispensable. More recently, Igarashi et al. (272) deleted both ori_s sequences with little effect on viral yields or viral DNA accumulation in infected cells. The results indicate that none of the three origins is uniquely required for viral replication. Rather, if an origin is required for either initiation or maintenance of DNA synthesis, any one of the three origins will most likely suffice.

All three origins, ori_L and the two ori_s, are situated between transcription initiation sites. The locations of the origins suggest that initiation of DNA synthesis might be *trans*-activated or at least enhanced by the changes in the local environment of the DNA due to transcription initiation events. In addition, it has been reported that a transcript designated as Ori_sRNA originates in the domain of the α22 and α47 genes and runs across the origins coterminally with α4 mRNA (268). The Ori_sRNA is detected at 9 hr and later postinfection, i.e., coincident with peak rates and termination of synthesis of viral DNA. The initiation of Ori_sRNA transcription is imprecise. A sequence $[N(GTGGGTGGG)_2(N \leq 10)]$ overlapping with the site of initiation of synthesis of the bulk of Ori_sRNA binds a cel-

lular protein with an unusual property; the protein binds in a sequence-specific fashion the above sequence in the context of single- or double-stranded DNA or RNA. The cognate sequence is not present elsewhere in the viral genome, and the DNA binding activity is present in a variety of tissues (400,571). The significance and function of the Ori_sRNA or of the cellular activity described above are unknown.

A central question that concerns the origins is why there are three of them. Also, it seems appropriate to wonder why the origins are distributed in both components and quasi-symmetrically, i.e., in the middle of the L component and in the sequences flanking the S component, when in fact one origin appears to suffice. The number and distribution of origins are particularly puzzling because the bulk of the DNA is replicated as a rolling circle, obviating the need for *de novo* initiation of viral DNA synthesis at an origin each time a copy of viral DNA is made.

Viral Proteins Involved in DNA Metabolism

Herpes simplex virus specifies a large array of proteins involved in nucleic acid metabolism and DNA synthesis. These proteins fall into two categories, i.e., proteins that are essential for viral origin-dependent amplification of DNA, and enzymes involved in nucleic acid metabolism [e.g., TK, ribonucleotide reductase, deoxyuridine triphosphatase (dUTPase), uracil glycosylase, and alkaline exonuclease] which for the most part appear not to be essential for viral growth in cells in culture. Contrary to early reports, topoisomerases do not appear to be encoded by HSV-1; the virus utilizes the p170 form of the host topoisomerase II (156).

Viral Proteins Essential for Origin-Dependent DNA Synthesis

Much of the initial evidence for viral proteins essential for DNA synthesis emerged from studies of the defects in DNA$^-$ ts mutants. More recently, the genes whose products are essential for DNA synthesis were identified by transfecting cells with a plasmid containing an origin of DNA synthesis and various fragments of the HSV genome. These studies identified seven genes mapping in the L component (open reading frames U_L5, 8, 9, 29, 30, 42, and 52) required for viral origin-dependent DNA synthesis. The seven genes specify a DNA polymerase (U_L30) with an apparent molecular weight of 140,000 (86,103,237,259,305, 306,500), a single-strand specific DNA-binding protein designated as ICP8 (U_L29) with an apparent molecular weight of 124,000 (84,99,109,287,387,394,500,525,580, 745), a protein binding to as many as three sites at or near the origin of viral DNA synthesis (U_L9) (160,161,323,324, 465) with a translated molecular weight of 94,000 (161), a protein which binds to double-stranded DNA and confers processivity on the DNA polymerase (U_L42) with a molecular weight of 62,000 (389,402,475,745), and three additional proteins (U_L5, predicted molecular weight of 99,000; U_L8, predicted molecular weight of 80,000; and U_L52, predicted molecular weight of 114,000). These three proteins form a complex in which each protein is present in equimolar ratios and which functions as a primase and, in the presence of ICP8, also as a helicase (119).

The DNA polymerase, the product of U_L30 ($ß_2$) in particular has been the object of numerous studies because of its unusual sensitivity to a variety of compounds [e.g., phosphonoacetate (PAA) and phosphonoformate]. In HSV DNA polymerase, *ts* mutants have been described (87,103,516, 517), and some have been found to be resistant to a variety of drugs inhibitory to wild-type viruses, including PAA (87,287,517) and nucleoside analogs (e.g., acycloguanosine) (118). The DNA polymerase forms a complex (1:1) with the product of the U_L42 gene (244).

ICP8 ($ß_1$) has also been extensively investigated, particularly by Knipe et al. (204,217–219,352,353,525,526) and by Ruyechan (579,581,583). The protein has an apparent molecular weight of approximately 120,000. It has an affinity for and binds cooperatively to single-stranded DNA (518,580). The *ts* mutants in this gene fail to synthesize viral DNA at the nonpermissive temperature (108,219), as do deletion mutants in cells which do not provide ICP8 in *trans* (468). ICP8 appears to be essential in anchoring the polymerase to the replication complexes and appears to interact specifically with U_L9 origin-binding protein (52,204). The protein promotes renaturation of cDNA strands and strand transfer—a key function for a high level of recombination in infected cells (55,154).

Dimers of the origin-binding protein (U_L9) bind to the sequence CGTTCGCACTT or its derivatives at three unequal sites with decreasing affinity (159,324). The sites flank the AT-rich sequences in the origins. The binding is cooperative, and the order of binding to these sites (designated as I, II, and III) reflects the affinity of the protein for each site. The bound U_L9 protein binding to two sites loops and distorts the intervening AT-rich sequences (323). The looping is independent of the phasing of the binding sites. In contrast, the required distortion is dependent both on the position of the binding sites and the free energy of supercoiled DNA. The origin-binding protein has been shown to be an ATP- or dATP-dependent helicase, unwinding DNA in the 3′ to 5′ direction. The helicase activity is increased in both rate and extent by ICP8 (52).

Relevant to the role of U_L9 and the origins of HSV DNA replication is the report that transfection of cells with plasmids encoding six (U_L5, U_L8, U_L29, U_L30, U_L42, and U_L52) of the seven proteins essential for viral replication is sufficient to induce the amplification of SV40 DNA integrated into cellular chromosomes but not HSV DNA synthesis (241). The U_L9 gene was dispensable. While the results are convincing, the interpretation of these results is far from clear. The key issue is whether cellular pro-

teins (or T-antigen) direct the viral gene products to specific sites analogous to the function attributed to U_L9 or whether the viral proteins produced changes in cellular chromosomes that resulted in cellular DNA synthesis. While the Heilbronn et al. (241) report could be construed as supporting the hypothesis that the U_L9 protein modifies the AT-rich sequence at the origin and enables the assembly of the proteins required for initiation of viral DNA synthesis, it casts a pall on the notion that the seven viral proteins listed above are all that is required for viral DNA synthesis. If, in fact, the six proteins encoded by U_L5, U_L8, U_L29, U_L30, U_L42, and U_L52 interact with cellular proteins to amplify the cellular DNA, the failure to replicate viral DNA *in vitro* by the seven proteins cannot be attributed solely to failure to find the right buffer composition for the reaction mixture.

Proteins Involved in Nucleic Acid Metabolism and Not Essential for Viral DNA Synthesis in Cells in Culture

Other proteins undoubtedly play a role in processing, cleavage, and packaging of the genomic viral DNA, as well as in the production of precursors of DNA synthesis; for example, as described below, the alkaline DNAse is not among the seven essential genes and appears to play no role in DNA synthesis (724), notwithstanding reports to the contrary (194,443).

Alkaline DNAse activity in infected cells was first reported in 1963 (306). The gene has been mapped by transient expression in oocytes (503) and by the use of HSV-1 × HSV-2 recombinants (26) to a site corresponding to U_L12 (402). The protein is encoded by a 2.3-kb mRNA (150) and has a predicted translated molecular weight of 67,503 (402) and an apparent molecular weight of 80,000 to 85,000 (27). It has been reported that U_L12 is required for egress of capsids from the nucleus (612). While it can be rationalized that the function is required to enable capsids to make their way through marginated chromosomes at the inner nuclear membrane, it would be useful to know whether the mutation on which this conclusion is based does not affect the expression of a U_L11 since deletion mutant in U_L11 had a similar phenotype (20). The alkaline DNAse accumulates in large amounts in nuclear dense bodies shown to contain proteins derived from the nucleolus (524).

Thymidine kinase is one of the most studied viral proteins. A unique characteristic of TK is that its substrate range is far greater than that of its host counterpart. Although it has been designated as a deoxypyrimidine kinase, it in fact phosphorylates purine pentosides and a wide diversity of nucleoside analogs that are not phosphorylated efficiently by cellular kinases (281,310,318). This characteristic of TK is the basis for the effectiveness of various nucleoside analogs in the treatment of experimental and natural herpesvirus infections. The observation that TK is essential for normal virus multiplication in experimental infections (186,687) but not in cell culture (311) is the basis of much of the probing of the HSV genome structure done in recent years (490,498,499). Mutants in the *tk* gene fall into several groups. Some fail to produce functional TK altogether, whereas others either make reduced amounts of enzyme or an enzyme with an altered substrate specificity which is resistant to the analog used in the selection process (126,186,502,674).

The ribonucleotide reductase encoded by HSV-1 consists of two proteins. The large subunit, ICP6 (260,261,270, 510) has an apparent molecular weight of 140,000 and a predicted translated molecular weight of 124,043 (402). The small subunit has an apparent molecular weight of 38,000 (16,510) and a predicted translated molecular weight of 38,017 (402). The two proteins are encoded by 3′ coterminal mRNAs of 5.0 kb for the large subunit and 1.2 kb for the small subunit (12). The two proteins are tightly associated in a $\alpha 2 \beta 2$ complex (16,17,275), and both subunits are required for activity (16,191,264). Ribonucleotide reductase functions to reduce ribonucleotides to deoxyribonucleotides, creating a pool of substrates for DNA synthesis. The viral enzyme is not essential for growth in actively dividing cells maintained at 37°C (123,220). However, it is required for efficient viral growth and DNA replication in nondividing cells or in cells maintained at 39.5°C (221,510), indicating that, at 37°C, actively dividing cells can complement the viral function.

The uracil DNA glycosylase encoded by HSV presumably functions in DNA repair and proofreading. Uracil DNA glycosylase acts to correct insertion of dUTP and deamination of cytosine residues in DNA; the extremely high G+C content of HSV DNA makes this an important element of error correction in HSV DNA replication. The HSV-induced uracil DNA glycosylase has been identified by Caradonna and Cheng (78), and its coding domain was initially mapped to between 0.065 and 0.08 map units (79), corresponding to the U_L2 open reading frame (402). Subsequent *in vitro* translation experiments definitively identified U_L2 as the uracil DNA glycosylase gene (744). The protein has an apparent molecular weight of 39,000 (79) and a predicted translated molecular weight of 36,326 (402). The gene has been deleted and is nonessential for growth of the virus in culture (446).

Deoxyuridine triphosphate nucleotidohydrolase (dUTPase) acts to hydrolyze dUTP to dUMP, providing both a mechanism to prevent incorporation of dUTP into DNA and a pool of dUMP for conversion to dTMP by thymidylate synthetase. An HSV-encoded dUTPase has been identified (78,740); contrary to early reports (740), the purified enzyme is specific for the hydrolysis of dUTP (737). The viral gene has been mapped to 0.69 to 0.70 map units by transient expression (509), corresponding to the U_L50 open reading frame (402). In HSV-1(17)*ts*K, a mutant in ICP4 used for analyses of gene regulation, dUTPase activity appears to be lacking (127). The dUTPase gene has

subsequently been shown to be nonessential for growth of the virus in tissue culture (187).

Assembly of Capsids

Capsids are assembled in the nucleus (Figs. 1 and 4). The steps in the assembly have not been defined, although capsids have been assembled *in vitro* from capsid proteins made in insect cells by baculoviruses carrying the genes specifying capsid proteins (691). Viral DNA is packaged into preformed capsids containing the protease specified by U_L26 and the scaffolding protein (the products of self-digestion of the U_L26 protein and of the substrate specified by $U_L26.5$ (144,368–371).

The assembly of full capsids containing DNA requires the participation of numerous noncapsid proteins as well. These include at the very least the products of U_L6, U_L15, U_L25, U_L28, U_L32, U_L33, U_L36, and U_L37 genes (8,10,96, 189,495,539,618,619,686).

Cleavage and Packaging of HSV DNA

Newly synthesized viral DNA is "processed" and packaged into preformed capsids. "Processing" involves amplification of *a* sequences and cleavage of viral DNA lacking free ends, i.e., in circular or head-to-tail concatemeric form. Associated with the processes of DNA replication, cleavage, and packaging is the isomerization of the DNA. There is considerable genetic evidence that cleavage and packaging of DNA are linked processes (344,345). The data on the isomerization of the DNA has come from three sources: (a) analyses of the termini of standard viral genomes (129,436,438), (b) analyses of termini of viral genomes containing insertions of additional *a* sequences (98,435,701), and (c) studies on amplicons (plasmids containing an origin of viral DNA synthesis and one or more *a* sequences which are amplified and packaged with the aid of a helper virus) (133,134,638,704,705).

The net result of the process of cleavage of standard genomes from concatemers is the generation of a free S component terminus consisting of one *a* sequence with a terminal DR1 sequence, containing only a single base pair and one 3′ nucleotide extension (436), and a free L component terminus, consisting of one to several directly repeated *a* sequences and ending in a DR1 containing 18 bp and one 3′ nucleotide extension. Upon circularization of the DNA following entry into cells, the two partial DR1 sequences together would form one complete DR1 shared by two *a* sequences. In the reverse process of linearization of viral DNA for packaging, cleavage of endless (circular or concatemeric) DNA occurs asymmetrically within a second DR1 distal from the *c* sequence and, in an ideal case, shared by two *a* sequences. Junctions containing a single *a* sequence are cleaved (134). The results of such studies have been interpreted to indicate either that the sequence *xay* is cleaved to yield *xa* and *y* and the *y* product is processively degraded along the DNA to the next *a* sequence or that the cleavage simultaneously yields both *xa* and *ay* by amplification of the *a* sequence during the cleavage process (133,134,701). Parenthetically, there is little doubt that DNA lacking a terminal *a* sequence could be degraded, but inasmuch as nearly 50% of the L-S component junctions are of the *bac* type, i.e., have a single *a* sequence, a hypothesis whose logical extension is that 50% of newly synthesized DNA is degraded during packaging, does not make biologic sense.

Deiss et al. (133) analyzed the cleavage and packaging of a series of amplicons. Those lacking the U_b sequence were amplified and packaged, but they acquired an intact *a* sequence from the helper virus. Those lacking the U_c sequence were not subject to cleavage-packaging. Furthermore, U_b and U_c contain domains conserved in several herpesviruses and which were designated *pac1* and *pac2*, respectively. The model (133) that best fits the data, presented here in a slightly modified form (Fig. 10), consists of several steps: (i) a cleavage-packaging protein attaches to the U_c sequence; (ii) a putative structure on the surface of the capsid complexes with a U_c bound protein sequence, loops the viral DNA, and scans from the bound *a* sequence (a_i) across the L component toward the end of the S component until it detects the first U_c domain of an *a* sequence in an identical orientation; (iii) in the juxtaposed *a* sequences, the DR1 sequence of one *a* is cleaved and the gap is repaired, resulting in the generation of an *a* sequence by the mechanism proposed by Szostak et al. (679) to explain recombinational events resulting in gene conversion; and (iv) cleavage then occurs in the DR1 shared by the two *a* sequences. In this model, the *a* sequences in the internal inverted repeats play no role in the packaging of the unit-length molecule, consistent with the observation that HSV-1 DNAs from which the internal inverted repeats are deleted do package effectively.

The packaging component of the model of Deiss et al. (133) predicted that the length of the packaged DNA be defined by the distance between two directly repeated *a* sequences. However, defective genomes consisting of 17+ direct reiterations of a unit consisting of an Ori_s and an *a* sequence are readily detected in virions of HSV stocks derived by serial passages at relatively high multiplicities (196). These observations are consistent with the hypothesis that, besides the scanning mode, there is a "head-full" recognition element which selects the juxtaposed *a* sequence once a threshold amount of DNA has been packaged. Shorter fragments of HSV DNA are packaged into capsids, but these capsids do not become enveloped (705). A hypothesis that may explain the apparent contradiction is that packaging aborts when the DNA reeled into the capsid is smaller than full length, but that the capsid does not disgorge the packaged fragment.

The viral proteins responsible for the cleavage-packaging event have not been identified. However, (a) Chou and

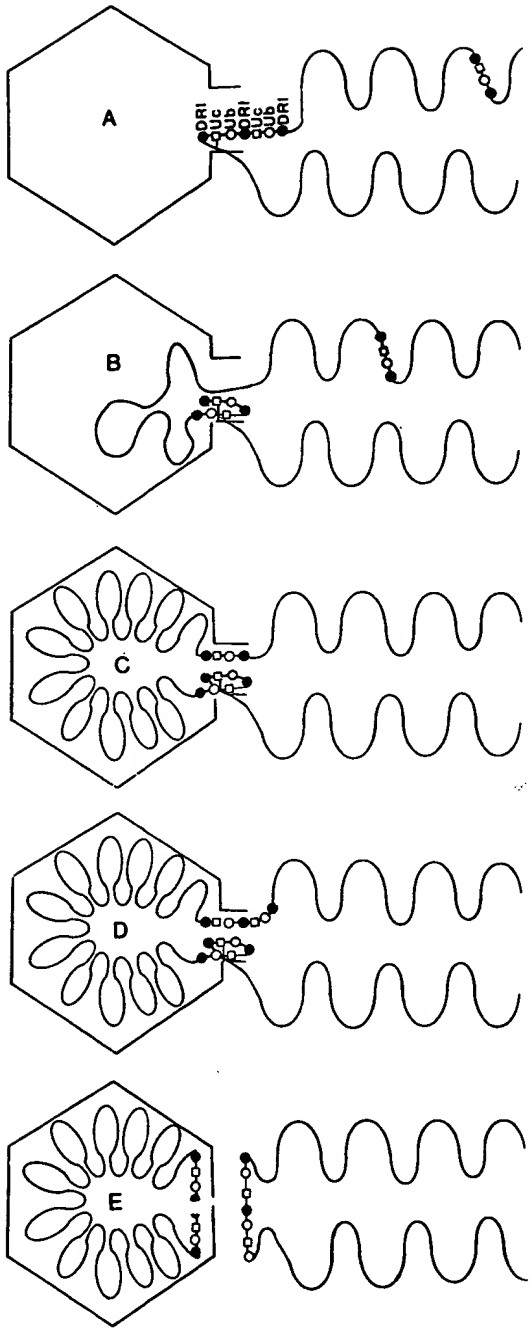

FIG. 10. Packaging of HSV-1 DNA. The current model developed by N. Frenkel and associates is described in Deiss et al. (133) and in the text. The model requires that (a) proteins attach to components of the *a* sequence, probably U_c; (b) empty capsids scan concatemeric DNA until contact is made in a specific orientation with the first protein-U_c sequence (capsid A); the DNA is than taken into the capsid B until a "head full" or contact is made with an *a* sequence whose nucleotide arrangement is in the same orientation (i.e., one genome equivalent in length away) is encountered (capsid C); the packaging signal requires nicking of both strands from signals on opposite sites of a DR1 sequence. In the absence of two adjacent *a* sequences (capsid D), the juxtaposition of the *a* sequences would results in duplication of the *a* sequence (capsid E), as described by Deiss et al. (133).

Roizman (96) have identified two viral proteins that form a sequence-specific complex with the portions of the U_c sequence containing *pac2*, (b) capsids contain a protein which binds viral DNA (VP19C or ICP32) (60), (c) Wolhtrab et al. (741) reported that the *a* sequence is specifically cleaved by virus-induced endonuclease, and (d) Table 1 lists numerous genes whose products appear to play a role in the cleavage-packaging of viral DNA.

Inversions of the L and S Components

The isomerization of HSV DNA resulting from the inversion of the L and S components relative to each other is an intriguing, tantalizing feature of the HSV genomes shared with only a few other herpesviruses.

In its circular form, the HSV genome forms two isomers, each containing two L-S component junctions. Cleavage of one circular isomeric form at the two junctions would yield the P and I_{SL} arrangements, whereas the corresponding cleavages of the other circular isomer would yield the I_S and I_L isomers. Generation of the I_S and I_L arrangements from the first circular isomeric form would require inversion of either the S or the L component through the inverted repeat sequences.

Fundamentally, there are several issues. First, inversion of covalently linked components is not a property of all herpesvirus genomes. Second, the physiologic function of the inversions is not clear inasmuch as genomes frozen in one orientation as a consequence of deletion of internal inverted repeats are viable (286,490). However, all wild-type isolates examined to date do contain the inverted repeat sequences, and viruses lacking internal inverted repeats have a reduced capacity for growth in animal tissues. Third, insertion of the junction between the L and S components, and especially of the 500-bp *a* sequence, results in additional inversions of DNA segments contained between inverted repeats of *a* sequences (98,435,436,438). Deletion analyses have shown that inversions are associated with the sequences DR2 and DR4; deletion of these sequences results in a gross reduction in the inversion frequency (98). Lastly, inversion of viral DNA segments flanked by other domains of the genome or inversion between repeated foreign DNA sequences was observed in some instances but not in others. In the case of fragments duplicated in different components of the HSV DNA, the segment of the genome flanked by the inverted repeats does not invert (435,498). DNA fragments flanked by inverted repeats contained in the same component do invert. In some instances, the inversions were accompanied by a high-frequency gene conversion (492). Weber et al. (717) reported that inversions of DNA segments flanked by inverted Tn5 transposon elements resulted from recombination events through homologous sequences and was not the consequence of a recombinational event mediated at a specific *cis*-acting site by *trans*-acting viral proteins. Thus, inversions of ampli-

fied DNA sequences flanked by inverted Tn5 sequences at least 600 bp or longer were noted in cells transfected with the genes specifying the seven proteins required for viral DNA synthesis. As in the case of amplicons containing two *a* sequences in an inverted orientation (436), inversions were not observed in the absence of DNA synthesis.

The central issue is not that DNA sequences flanked by inverted repeats tend to invert as a consequence of homologous recombination, but rather the frequency of such inversions and the specificity of the process in infected cells. The DNA extracted from a plaque generated by a single virus particle, presumed to be in one arrangement of DNA, contains all four isomers of HSV DNA in equimolar concentrations. In the case of DNA segments flanked by inverted repeats of nonjunction fragments, the fraction of the genomes showing inversions even after many serial passages is seldom more than a small fraction of the total. A more careful analysis by Dutch et al. (153,155) showed that DNA flanked by inverted *a* sequences had a higher rate of inversion than DNA flanked by non-*a* sequences of equal length. These studies also demonstrated that the sequence required for this process was a 95-bp sequence containing DR1 and U_c. Bruckner et al. (61) reported the partial purification of an activity mediating *in vitro* recombination between repeated copies of the *a* sequence and concluded that the recombination proceeds by a site-specific mechanism.

Viral Membrane Proteins, Virion Envelopment, and Egress

Appearance of Modified Nuclear Membranes in Infected Cells

The hallmark of infected cells late in infection is the appearance of reduplicated membranes and thick, concave or convex patches, particularly in nuclear membranes (Fig. 6) (111,166,356,401,439,454,600,601,624,743). Nuclear envelopment takes place at these patches. Because the enveloped virions do not contain detectable amounts of host membrane proteins, it is likely that the patches represent aggregations of viral membrane proteins, presumably including the viral glycoproteins on the outside surface and anchorage and tegument proteins on the inside surface.

Processing of HSV Glycoproteins

The general pattern of the biosynthesis of herpesvirus glycoproteins appears to follow that of eukaryotic cell glycosylated proteins (74,640). Specifically, nonglycosylated precursors of herpesvirus membrane proteins are synthesized on polyribosomes bound to the rough endoplasmic reticulum. Glycosylation includes translational and posttranslational events. Thus N-linked glycosylation is initiated by transfer of preformed glycans [$(glucose)_3$-mannose-$(N\text{-acetylglucosamine})_2$] from a dolichol phosphate lipid carrier to asparagine residues in the sequence Asn-X-Thr/Ser (X can be almost any amino acid) of a nascent polypeptide (362,391,680). During transit through the Golgi apparatus, the oligosaccharide chains are trimmed by glucosidases, mannosidases, and so forth to yield a polymannosyl chain frequently referred to as the high-mannose glycans (267,649). The high-mannose glycans are frequently converted by glycosyl transferases to complex glycans which consist of a pentasaccharide core [$(mannose)_3$-$(N\text{-acetylglucosamine})_2$] and a number of side chains (antennae) with the composition of sialic acid-galactose-glucosamine. Fucose, when present, is usually added to the completed side chains (267). O-linked glycosylation occurs less frequently than N-linked glycosylation (74,292,453,466,620,640); it is initiated by the transfer of N-acetylgalactosamine to the hydroxyl group of threonine or serine and is followed by the addition of galactosamine, N-acetylglucosamine, fucose, and sialic acid in the Golgi apparatus (38). The extent of glycosylation and the structure of complete glycans is affected by the conformation of protein around the glycosylation site, inasmuch as much as the structure affects access by enzymes involved in processing. Conformation of the protein may also explain the heterogeneity of glycans attached to a given protein.

Current information on the structure of the HSV glycans has been summarized in detail elsewhere (74,640). Thus N-linked high-mannose, O-linked, and complex heterogeneous glycans have all been reported to exist in glycoproteins specified by HSV-1. There is no evidence to date that processing of nonglycosylated precursors to the fully glycosylated stage requires the synthesis of virus-specified enzymes, although the data do not specifically exclude the possibility that the virus encodes at least some enzymes whose functions are similar to those of the host.

Nothing is known regarding the function and requirements for O-linked glycosylation of herpesvirus glycoproteins. N-linked glycosylation is required for infectivity, inasmuch as blocking of N-linked glycosylation by tunicamycin blocks the accumulation of glycosylated proteins and of enveloped virus (480,485,487). Conversion of high-mannose glycans into complex-type glycans appears to be required for the egress of the virus from the infected cell but not for infectivity (72,291,327,611).

There is considerable evidence that, after synthesis, the viral glycoproteins are transported to the plasma membrane and can be found in all cytoplasmic membranes of the cell. The viral glycoproteins in the cellular membranes are major targets of the immune response to the virus. Several authors reported that, when viral glycoproteins are specified by genes resident in the environment of the cells, maturation and transport occur faster than when the proteins are specified by genes resident in the viral genome and are expressed during infection (14). The difference may simply reflect timing and intracellular traffic congestion. In cells expressing both a glycoprotein gene resident in the cellu-

lar genome and the corresponding gene resident in the viral genome, the former gene is expressed earlier; the glycosylation and transport of this protein does not compete with that of abundant viral glycoproteins made later from transcripts of genes resident in the viral genome.

Properties of Minor Glycosylated and Nonglycosylated Membrane Proteins

McGeoch et al. (402) predicted that the products of U_S9, U_L10, U_L20, U_L43, and U_L53 are membrane proteins. U_L53 has been shown to encode glycoprotein K (271,529). U_L10 encodes an abundant dispensable glycoprotein (gM) associated with both cellular membranes and virion envelopes (19,22). Current studies (J. Baines and B. Roizman, in preparation) suggest that the protein contains several transmembrane domains and that at least two short loops accessible to proteases project from the surface of the cell. One of the loops contains a single glycosylation site which is apparently used. Deletion mutants are impaired in growth properties.

U_L20 is dispensable. In cells infected with wild-type virus, the product of the gene is made in relatively small amounts; it is highly hydrophobic, appears to be associated with membranes, and does not appear to be glycosylated (23,712). Preliminary observations suggest that it localizes in nuclear membranes, the intermediate compartment, and the Golgi but not in the plasma membrane. The properties of the deletion mutant are described below.

U_L43 presents a special problem in that the domain of the gene is poorly defined. The transcription initiation site is unknown, and the TATAA box is only a few nucleotides away from the putative translational initiation site. MacLean et al. (385) reported that the open reading frame as defined by McGeoch et al. (402) is dispensable without effect on viral replication. L. Kaplan, D. Barker, and B. Roizman (unpublished results) also deleted the sequences assigned to this open reading frame. Although a gene product could not be demonstrated by immunoblotting of lysates of cells infected with a mutant containing an in-frame insertion of an epitope, as has been done for other open reading frames (95,371), immunofluorescence studies demonstrated the presence of an antigen which appeared to be associated with Golgi (P. L. Ward and B. Roizman, in preparation).

In addition to these proteins, two others have recently been shown to associate with membranes. The product of the membrane protein encoded by U_L34 has been described above in connection with its phosphorylation by the U_S3 protein kinase. The association of the U_L24 gene product with membranes is based on the observation that deletions within the domain of the gene cause infected cells to fuse (280). Several lines of evidence suggest that HSV membrane proteins form specific complexes. The existence of one complex, the Fc receptor formed by glycoproteins E and I, can be deduced from the observation that monoclonal antibody to either precipitates both glycoproteins (288). Similarly, it has been reported that gH associates with gL (U_L1) (271).

Nothing is known of the mechanism by which viral glycoproteins enter nuclear membranes. One facile (and possibly incorrect) explanation is that viral membrane proteins are made on rough endoplasmic reticulum and are translocated laterally to the contiguous nuclear membrane.

Envelopment

Nuclear DNA-containing capsids attach to patches of modified inner lamella of the nuclear membrane and become enveloped in the process. The emphasis on "DNA-containing" stems from electron microscopic observations which show that envelopment of empty capsids occurs rarely, although there is no evidence that "full" capsids always contain a full-length molecule of HSV DNA (557). As discussed above, Vlazny et al. (705) demonstrated that capsids containing fragments of HSV DNA less than standard genome length are retained in the nucleus. A plausible explanation for this phenomenon is that capsids containing DNA become modified and acquire affinity for membrane tegument structures in nuclei. Conceivably, capsids totally lacking this putative modification are unable to bind to the underside of the thickened patches containing viral proteins in the nuclear membranes.

There is general agreement that the inner lamella is the site of initial envelopment (Fig. 6). However, even cursory examinations of thin sections of infected cells elicits the rediscovery that envelopment occurs in the cytoplasm since the cytoplasm abounds in capsids juxtaposed to patches of modified cytoplasmic membranes in the process of envelopment or deenvelopment. Stackpole (650) is the originator of the idea that the capsids become enveloped at the inner lamella, deenveloped at the outer lamella, reenveloped by the endoplasmic reticulum, and released in the extracellular environment either by envelopment at the plasma membrane or by fusion of vesicles carrying enveloped virus at the plasma membrane. The large number of particles seemingly undergoing cytoplasmic envelopment makes a strong case for this model. However, thin sections showing capsids being enveloped at the nuclear membranes are extremely rare, suggesting that the process of envelopment is very rapid. Since every capsid undergoing putative envelopment in the cytoplasm must have been enveloped and deenveloped in transit through the nuclear membranes, the disparity in the numbers of capsids being enveloped at nuclear and cytoplasmic membranes suggest that either (a) the rate of envelopment at the nuclear membranes is significantly faster than that at the cytoplasmic membranes or (b) that the capsids in juxtaposition to cytoplasmic membranes are artifacts and represent capsids that are deenveloped or arrested in their movement through membranes. The key question of whether the cytoplasmic, semienveloped cap-

sids are in fact in the process of being enveloped rather than arrested, i.e., transient structures resulting from deenvelopment, cannot be answered by electron microscopic snapshots anymore than the plot of a motion picture can be deduced by viewing the prints of a few random frames. It is of interest to note that electron micrographs published in a recent study which strongly defended the serial envelopment hypothesis showed that some of the capsids undergoing cytoplasmic envelopment were in fact partially degraded, as evident from the protruding DNA (726).

One hypothesis for the abundance of partially enveloped structures at cytoplasmic membranes is that virions contained in the endoplasmic reticulum attach to receptors and reinfect the cell from within, but unlike capsids which become deenveloped at the plasma membrane during entry, the capsids entering the cytoplasm by fusion of envelopes with membranes of transport vesicles may not transported to the nuclear pore. Thus the capsids deenveloped at the plasma membrane are most likely transported to the nuclear pores by specific elements of the cytoskeleton. These connections may not be available for capsids entering cytoplasm through transport vesicles. It is of interest that cytoplasmic semienveloped capsids are prevalent in continuous cell lines but less frequent in infected primary human diploid cells (B. Roizman, unpublished observations).

Translocation of Virions Across the Cytoplasm to Extracellular Space

Following envelopment, virions accumulate in the space between the inner and outer lamellae of the nuclear membrane. In the cytoplasm, intact enveloped particles are usually seen inside structures bounded by membranes (128,601). This observation is not surprising, inasmuch as structures bounded by membranes with surface glycoproteins are not likely to fare well unprotected in the cytoplasm. In two-dimensional sections, these structures appear to be vesicles; in a few electron micrographs, tubular structures have been seen, and the probability exists that, in some cells, the cisternae of the endoplasmic reticulum extend to the plasma membrane (601).

There is a general expectation that (a) virions must pass through Golgi as they traverse the cytoplasm and (b) the sorting of virions is mediated by cellular machinery. Indeed, Johnson and Spear (291) proposed, in part on the basis of studies done with monensin, that virions are secreted via the Golgi apparatus following a pathway similar to that taken by secreted soluble proteins. Several observations suggest that the situation may be more complex and that HSV controls and at the very least directs traffic through the infected cell.

1. In cells defective in processing of high-mannose to mature glycoproteins, infectious virions accumulate but are not transported into the extracellular space (25,72).
2. Virions made in cells infected with mutants in gH and carrying the mutated glycoprotein are not translocated into the extracellular space. Conversely, the translocated virions do not contain gH (142). It is tempting to deduce from this study that the mutated gH exhibits a retention sequence which precludes the translocation of the virion.
3. Deletion mutants in U_L20 replicate and form plaques in human 143 cells but not in Vero or HEp-2 cells. In Vero cells, infectious virus accumulates in the space between the inner and outer lamellae, and transport vesicles normally associated with virion traffic across the cytoplasm are lacking. Moreover, U_L20^- virus does not form plaques, which is concordant with the absence of virions in the extracellular space (23). Additional studies showed that (a) purified virions were significantly enriched with high-mannose glycoproteins; (b) the total cell glycoproteins exhibited a ratio of fully processed to high-mannose glycoproteins similar to those of wild-type infected cells, and the processed glycoproteins contained terminal sialic acid, suggesting that the viral glycoproteins not associated with virions are sorted at least through the *trans*-Golgi; and (c) infected cell plasma membranes contained significantly less glycoprotein than cells infected with wild-type virus (15).
4. Virions in or associated with Golgi have been seen but rarely. In light of the large number of virions produced in the infected cells, either the processing through Golgi is extremely rapid, or Golgi enzymes are translocated from the Golgi to the transport vesicles. Part of the solution of this riddle may stem from the observation that the Golgi is fragmented and dispersed throughout the cytoplasm in infected Vero or HEp-2 cells (69). The evidence suggests that U_L20 is essential primarily in cells in which the Golgi is fragmented and that, in these cells, it enables the traffic from the outer nuclear membrane to the Golgi and from the *trans*-Golgi to the plasma membrane.
5. Both gE and gI, besides being required for entry through the membranes of polarized epithelial cells, also play an essential role in the basolateral spread of virus in those cells (A. Bloom, K. Rott, A. Sears, and B. S. McGwire, in preparation) and enhance cell-to-cell spread in nonpolarized cells (145). It is conceivable that the virus employs several different gene products to ensure full processing of glycoproteins and safe transport across the cytoplasm to the appropriate cell surface.

Superinfection of Infected Cells

In cells infected with a mutant mapping in the S component, but not in cells infected with wild-type virus, empty capsids accumulated in large numbers at the outer surface of the nuclear membrane, suggesting the possibility that HSV encodes a function to prevent reinfection of cells,

particularly with virus which had been released from those cells (693). This function has been attributed to gD (70,71, 73). Indeed, cells expressing gD allow attachment, but the virus is endocytosed and destroyed. Treatment of the cells with antibody to gD renders the cells susceptible to infection. Mutants capable of infecting these cells were readily isolated and were found to map foremost in gD, but also in other, as yet unidentified viral genes (56,70,73).

A most interesting, and potentially significant, finding was that clones of baby hamster kidney cells were found to vary with respect to their ability to express α genes, and indications are that they vary with respect to their susceptibility to infection. Mutations which confer the capacity to infect these cells, albeit at variable efficiencies, also map at least in part in gD (572).

The model that best fits the available data is that cells may express at least two surface receptors for entry of HSV into cells. One receptor has an affinity for gD and is sequestered by wild-type gD. In cells expressing gD, this receptor becomes available to incoming wild-type virus by pretreatment of the cells with antibody to gD. The other, secondary receptor does not interact with wild-type gD but has a low affinity for a specific set of mutated gD molecules. While the secondary receptor can be sequestered by mutated gD, the affinity of the secondary receptor for the mutant gD molecules may be lower than that of wild-type gD for the primary receptor. This may explain why cell lines expressing mutant gD are infectable by both wild-type virus (primary receptor is present) and viruses carrying mutated gD (low affinity favors reequilibration of the mutant gD in the presence of viral particles attached to the surface of the cells). In the highly restrictive clonal cell lines which do not express gD, the primary receptor may be absent or mutated. A component of this model is that other viral proteins (e.g., gB, gH, and so forth) affect the interaction of gD with cellular proteins and hence are subject to selection of mutations which increase the efficiency of viral entry into cells (56).

REGULATION OF VIRAL GENE EXPRESSION

A key feature of productive HSV infection is that the genes comprising the five groups, α, $ß_1$, $ß_2$, $γ_1$, and $γ_2$ are tightly regulated with respect to both abundance and timing of their expression. Herpes simplex virus genes function within the environment of eukaryotic nuclei, and by necessity they contain signals that enable their transcription by cellular factors. In addition, viral genes contain response elements *trans*-activated by viral gene products. The focus of this section is on the components of virus-specific regulation of gene expression.

Structure of HSV mRNAs

Herpes simplex virus DNA is transcribed by RNA polymerase II (115). Viral mRNAs are capped, methylated, and polyadenylated, although nonpolyadenylated RNAs of the same sequences can be isolated (18,30,31,626,628,668). Internal methylation is readily apparent in RNA made early but not late in infection (30). Notwithstanding the efficient expression of HSV genes in the environment of higher eukaryotic cells, only four open reading frames, α0, α22, α47, and U_L15, have been shown to yield spliced mRNAs. Minor subsets of the transcripts of mapping to the gC (U_L44) and polymerase (U_L30) genes are also spliced (49,200). Transcripts sharing 5′ and particularly 3′ termini have been described (707). Attention has also been drawn to multiple initiation sites for the transcription of selected HSV genes (200,448,614,715,756), to the common observation that RNAs may extend beyond the usual polyadenylation site (12,97,255,528), and to selective and possibly regulated use of alternative polyadenylation signals (418). In contrast with the orderly transcription of intact cells are the random initiations experienced by more than one laboratory in nuclear run-off transcription assays late in infection (217,721).

The abundance and stability of the various HSV-1 mRNAs vary (198,199,626,628). In general, mRNAs of α and ß genes appear more stable than those of γ genes (742). Viral mRNAs may persist in the cell after their translation ceases (293,328,329).

Environment of the Viral Genes

The open reading frames identified to date are embedded for the most part in domains exhibiting both virus/host-common (e.g., TATAA and CAAT, SP1 response elements, and so forth) and virus-specific *cis*-acting sites. The absence of TATAA boxes from some transcriptional units has been noted (e.g., $γ_1$34.5) (97).

Studies on the structure of HSV genes have focused on two specific objectives. The first concerned the minimal promoter domain and the *cis*-acting sites required for gene expression. The second objective was to identify the *cis*-acting sites which confer upon the reporter gene the capacity to be regulated as an authentic viral gene. Only a few HSV genes have been analyzed in sufficient detail to reveal and dissect the *cis*-acting signals embedded in them. The most thoroughly studied, and the one which has generated the most perplexing results, is the *tk* gene. The α4 gene (Fig. 11) and, to a much lesser extent, two representative $γ_2$ genes are also worthy of discussion, although in these instances the results are more novel than perplexing.

α4 Gene

In the α4 gene, the 5′ nontranscribed domain extending upstream from the cap site to nucleotide −110 is capable of imparting to a reporter gene the capacity to be transcribed efficiently in the absence of viral *trans*-activating factors (380,381,498). Other than the transcription

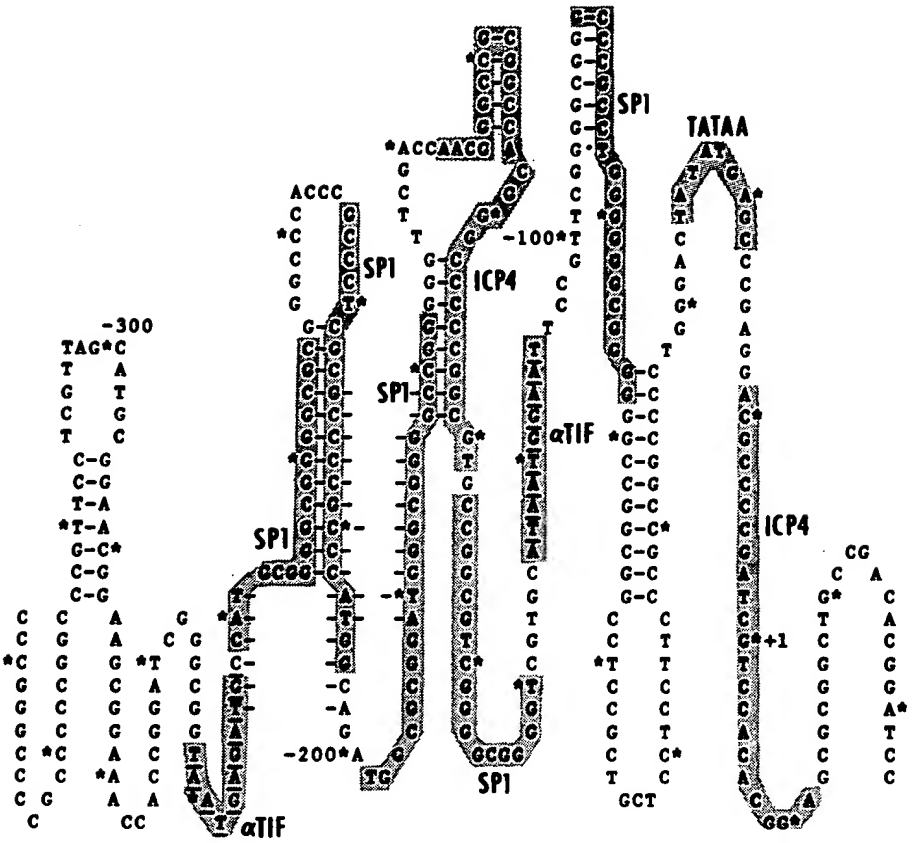

FIG. 11. Schematic representation of the structure of the 5′ nontranscribed domain of the α4 gene. The symbol * marks every tenth nucleotide. The transcription initiates at nucleotide −1. The shaded areas represent the binding sites of TATAA protein, SP1 and ICP4, and the host protein-αTIF complex. The sequence is shown in a form to emphasize the presence of the perfect G+C-rich inverted repeats that abound in this domain of the gene. In some instances, the G+C-rich regions can form alternate stem structures; these are identified by the dashed lines. Data from Mackem and Roizman (382).

initiation site, the *cis*-acting sites which affect expression have not been investigated in detail. The sequences upstream from nucleotide −110 confer on α and ß promoters a higher basal level of expression as well as the capacity to be induced as α genes by viral *trans*-activating factors (337). The higher level of expression conferred by the sequences upstream from nucleotide −110 is very likely due to the SP1 binding sites embedded in G+C-rich inverted repeats which abound in that region (296,297,337,380–382). At least one sequence which confers inducibility as an α gene is the *cis*-acting site for αTIF, i.e., 5′ NCGyATGnTAATGArATTCyTTGnGGG 3′ (337,380). Separation of minimal promoter and regulatory domains has been noted in other α genes (380–383). Although G+C-rich stretches are frequently found in α genes, SP1 binding sites have not been reported in other α genes (Fig. 11).

ICP4 binding sites have been reported in the promoter-regulatory domain, and across the transcription initiation site of the α4 gene, and in the promoter domain of the α0 gene, but not in the other α genes (176,335,336,447,559), and their significance is detailed below in the context of the function of ICP4.

In addition to the SP1 and αTIF-responsive *cis*-acting site, α gene promoters contain numerous other *cis*-acting sites for CAAT-box binding proteins, shown to affect gene expression markedly (648). The element GCGGAA, often present a variable number of times upstream of the transcribed domains of α genes, has been reported to bind proteins by footprint analysis (695). The role of these repeats and their environment may be deduced from the observation that the proteins purified by affinity to this sequence do not bind to a single isolated hexanucleotide (348). Complementary DNA clones that encode two distinct subunits of this transcription factor have been isolated; the amino acid sequence of one protein exhibits similarity to the Ets DNA binding domain (349). The function of the purine-rich GA element in the context of α gene promoters remains a mystery, however, inasmuch as mutations within this region of the α27 promoter in the context of the viral genome reduced the accumulation of α27-*tk* mRNA only slightly.

tk *Gene*

The initial analyses of the *tk* gene rested on the notion that it exemplified the structure of a typical eukaryotic gene. Only much later has there been an effort to understand its structure as a viral ß gene. The *tk* gene appears to have two transcription initiation sites, and mRNAs derived from both sites have been reported (141,505,756). The role of these sites in the expression of the gene in lytic infection is not clear. The 5′ nontranscribed region has been very thoroughly investigated by McKnight et al. (157, 410–414) and by Silverstein et al. (162,163,210). Initial studies identified a 110-nucleotide region upstream from the site of initiation of transcription that was minimally required for efficient expression in the absence of viral *trans*-activating factors. This promoter contains a "proximal" and two "distal" *cis*-acting sites, all of which are important for constitutive expression of the *tk* gene (756). In subsequent studies, a CCAAT box, two SP1 binding sites and an octamer motif (ATTGCAT) upstream of −116 were also identified (478). Mutations in all of these sites affect expression of the uninduced gene in rodent cells. However, sequence-specific mutagenesis of the octamer motif and of the SP1 binding sites indicated that they play a minimal role in the *trans*-activation of the *tk* gene by HSV infection, and attempts to define a *cis*-acting site that is virus-specific have not been successful (470,471). The protocol employed involved studies on linker scanning mutations. Inasmuch as the authors failed to identify mutations which affected *trans*-activation but not expression in the absence of viral *trans*-activating factors, they concluded that the viral factors which *trans*-activate viral ß gene expression act on a host factor and not directly on DNA.

More recent studies have focused on the interaction of ICP4 with domains of the *tk* gene and on mutations downstream from the *tk* cap site. Notwithstanding earlier reports that ICP4 does not bind to the *tk* gene directly (138,542), it is evident that the ICP4 does indeed bind to several domains of the gene, both upstream and downstream of the cap site, and that the binding sites include both sequences similar to the consensus binding site reported by Faber and Wilcox (177) and highly degenerate sites with little resemblance to the consensus (274,430). Of particular interest are the ICP4-binding sites in the transcribed noncoding domains and the role of the transcribed noncoding sequences on the regulation of *tk* as a ß gene, as discussed below.

γ *Genes*

The structure of the γ genes, and particularly of the γ_2 genes, is the least well understood and is likely to vary from gene to gene. Analyses of several genes, i.e., U_L44 (gC), U_L38, $U_L49.5$, and U_S11, suggest that the sequences required for efficient expression include the TATAA box and extend into the 5′ transcribed noncoding domain (114,141, 172,229,257,258,266,294,295,396,397,613,625). The sequences required for the γ_2 regulation of these genes also appear to include sequences downstream from the TATAA box. Embedded in transcribed domains as well as the 5′ nontranscribed sequences are ICP4 binding sites. Specific sequences which are required for efficient transcription have been reported within 5′ transcribed, noncoding domain of some but not all γ genes (229).

Trans-Activation of α Genes by αTIF

Background

The *trans*-activation of α genes by a putative structural component of the virus was first reported by Post et al. (498) in experiments designed to map the domains of the α gene promoters. In these experiments, transcription of a chimeric gene residing in L cells and consisting of the α4 gene transcribed noncoding and 5′ untranscribed domains fused to the transcribed noncoding and coding domains of the *tk* gene was induced by an infection with a tk^- virus in the presence of cycloheximide. Similar results obtained with other α gene promoters suggested the possibility that the inducing factor was brought into the infected cells with the virion (380–382). The compelling evidence that the *trans*-activator is a component of the virion which acts on a specific response element came from three sources. First, Mackem and Roizman (382) reported that all DNA fragments derived from α promoters that were capable of being induced contained a consensus sequence 5′ GyATGn-TAATGArATTCyTTGnGGG (noncoding) common to the regulatory domains of all α genes. They also reported that some promoters contained G+C-rich, perfect inverted repeats; some of these stretches were later shown to contain SP1 binding sites. Kristie and Roizman (337) showed that homologs of the consensus sequence imparted on chimeric constructs the capacity to be induced by the virion component, whereas the G+C-rich domains affected the basal level of expression of chimeric genes. Second, Batterson and Roizman (33) reported that ultraviolet light-inactivated virus was capable of inducing α-*tk* chimeric genes and that the *trans*-activator was a component of the virion tegument inasmuch as HSV-1(HFEM)*ts*B7, a ts mutant, at the nonpermissive temperature induced the α-*tk* chimeric genes even though the capsids accumulated at the nuclear pore and failed to release viral DNA into the nucleus. Lastly, Campbell et al. (76) mapped the gene whose product induced the α chimeric gene to U_L48. The nucleotide sequences of the HSV-1(F) and HSV(17)syn^+ U_L48 genes were reported (125,481). The product of this gene had been previously recognized as VP16 present in 500 to 1,000 copies/virion (242,643) and also as ICP25 (261,262). The name by which this protein is known is subject to considerable confusion largely because Dalrymple et al. (125) gave it still another designation based presumably on its

apparent molecular weight deduced from its migration in polyacrylamide gels. The designation given to it by Pellett et al. (481), αTIF, reflects its function as the *trans*-activator of α genes.

Properties of the αTIF Protein

A report from McKnight et al. (409), followed quickly by many others, established that αTIF forms complexes with DNA, but only in the presence of a cellular protein, Oct-1, or under conditions in which Oct-1 can participate in the reaction (212,460,461,504,648,695). Specifically, (i) *in vitro* synthesized, labeled VP16 was demonstrated to form a complex with unlabeled DNA in the presence of crude uninfected cell extracts or partially purified Oct-1 (212,409) and (ii) the complex was retarded by antibody to αTIF, but not by preimmune serum (212,409,504). The αTIF gene falls into the γ_1 kinetic class and is predicted to encode a maximum of 490 amino acids with a translated molecular weight of 54,000 and a minimum of 479 amino acids if the first in-frame methionine codon is not used or is used intermittently (125,481). The functional domains of αTIF have been mapped by several laboratories (2,3,226, 227,660,694) with the following results.

(i) Kristie and Sharp (338) demonstrated that, at very high concentrations, αTIF could form complexes with DNA in the absence of additional host factors.

(ii) The key domains of αTIF responsible for the formation of multiprotein complexes have been mapped to amino acid stretches. Region I consists of the most basic amino acid stretch located between residues 173 and 241, whereas region II lies between residues 378 and 389. Mutations in region I diminished the ability of the protein to interact with DNA in the absence or presence of the Oct-1 POU domain, suggesting that this region is required for and may be involved in binding DNA (660).

(iii) The αTIF interacts specifically with the Oct-1 POU domain, but it is incapable of interacting with a POU domain in which helix 2 of the Oct-2 protein has been substituted for the corresponding helix of Oct-1 (661). Point mutations within region II also completely destroyed αTIF-induced complex assembly (226). Region II appears to be the domain of αTIF responsible for specific interaction with the Oct-1 homeo domain (660). Mutational analysis also suggested that region II is primarily responsible for αTIF interaction with an additional host factor, although mutations in region I can also affect this interaction (660). Since αTIF-lacking region II has a reduced capacity to interact with DNA, it is conceivable that deletions in region II alter its conformation. Parenthetically, the entire carboxy-terminal acidic activating region of the protein is absent from the varicella-zoster virus homolog of the protein encoded by the ORF10 gene. The ORF10 protein has been reported to be incapable of forming multiprotein complexes with Oct-1 (406).

(iv) The domain responsible for the *trans*-activation of α genes is contained within the highly acidic carboxy-terminal 80 amino acids. Proteins which lack the *trans*-activation domain may retain their capacity to form complexes with Oct-1 and DNA. However, deletion of the domain responsible for complex formation obliterates the capacity of αTIF to *trans*-activate. The key evidence concerning the role of this region emerged from observations that a chimeric protein consisting of the DNA binding domain of the yeast transcriptional activator GAL4 fused to the C-terminal domain of the αTIF gene induced a reporter gene to a high level (586). Analysis of this region has revealed that a net negative charge contributed to, but was not sufficient for, transcriptional activation. The observations that transcriptional activators of several classes contained hydrophobic amino acids in patterns resembling that of αTIF, and that a phenylalanine residue at position 442 was markedly sensitive to mutation, suggested that the mechanism of transcriptional activation by αTIF could involve both ionic and specific hydrophobic interactions with target molecules (117).

(v) The identity of the interactive cellular protein has been the subject of numerous studies. Stringer et al. (669) reported that the αTIF activation domain bound strongly and selectively to the human and yeast TATA-box binding factor TFIID. Lin and Green (365) presented evidence that αTIF stimulates transcription in the absence of ATP by increasing the number of functional preinitiation complexes through recruitment of the general transcription factor TFIIB to the promoter. In both of the above studies, mutations in the αTIF activation domain which affected *trans*-activation also decreased the affinity of the protein for transcription factors (276,365,669). Liljelund et al. (364) also reported that αTIF alters the binding of the TATA box factor to its response element. Kelleher et al. (307) suggested that the direct target of αTIF is not a general transcription factor but rather another factor referred to as an "adapter" or a "coactivator," which forms a bridge to one of the general transcription factors. Recently, the gene for such a putative adapter molecule, ADA2, has been isolated from yeast on the basis of its interaction with the acidic activation domain of αTIF and was shown to respond to some but not all acidic activator domains (39).

(vi) Located in the tegument, αTIF is a structural protein. Weinheimer et al. (720) deleted the U_L48 gene encoding the αTIF using a cell line which produced the protein to propagate the mutant virus. In infected cells, the deletion mutant induced nearly normal

levels of viral DNA synthesis and capsid production, but the amounts of encapsidated DNA were lower, and the electron microscopic studies suggested that it was defective in other steps of viral maturation.

(vii) Attempts to dissociate the function of αTIF as a *trans*-activator of α genes from its as yet undefined function as a structural protein have run aground. A mutant with an insertion into the U_L48 gene was shown to abolish the ability of the protein to induce recorder genes but did not render the virus noninfectious, although its ability to multiply and spread at low multiplicity of infection was impaired (3). The virus stocks were characterized by high virion/plaque-forming units (pfu) ratios. In infected cells, the expression of α0 and α27 were reduced four- to fivefold, whereas that of α4 was not. Interpretation of these results presents three problems. Foremost, the high virion/pfu ratios may also be interpreted to indicate that the structural function of αTIF was compromised, and therefore the two functions of αTIF have not been resolved. Second, the promoter domains of α genes contain numerous *cis*-acting sites for a variety of transcriptional factors, and each gene is readily expressed in cultured cells, albeit at a reduced basal level, in the absence of αTIF. High multiplicity of infection could compensate for weak promoter activity. Lastly, a perhaps more worrisome problem is that a very much reduced level of *trans*-activation, which might not be detectable in transfected cells, would be sufficient to enable the virus to multiply. In light of these issues, the report that the virus appeared to be avirulent in mice, was able to establish latency in the trigeminal ganglion, and reactivated upon explantation is subject to conflicting interpretations (657).

(viii) Lastly, observation that VHS binds to αTIF and precludes its interaction with its response element has been suggested to indicate that αTIF modulates the activity of VHS during infection (630). Since αTIF and VHS enter the cell with the infecting virus at the same time, but perform their functions in different compartments of the cell, such regulatory functions are probably of no great significance in the initial stages of infection. The association of the two protein does solve an important riddle, i.e., why the massive quantities of αTIF made late in infection fail to induce α gene expression at that time.

Oct-1 Protein

The Oct-1 protein was independently discovered in several laboratories studying a variety of biologic systems and has been designated as NF-A1, OBP100, OTF-1, NFIII, origin recognition protein C, αH1, and Oct-1 (189,334,467, 655,671,672). Oct-1 interacts with the octamer motif of the *cis*-regulatory element in the promoters of ubiquitously expressed sequences such as those encoding the small nuclear RNAs and the histone H2b genes (343,392). The identical octamer motifs present in cell-specific immunoglobulin promoters are thought to interact with the transcriptional factor Oct-2 (594). Oct-1 has been demonstrated as a stimulatory replication factor in the adenovirus system (467,514).

A nearly complete cDNA cloned by Sturm et al. (672) led to the recognition that Oct-1 is a homeobox protein which, along with two other mammalian transcription factors, Pit-1 and Oct-2, and the product of the nematode *Caenorhabditis elegans* Unc-86 gene, helped define a new class of homeobox proteins called POU proteins (for Pit, Oct, Unc) (246). These four proteins share a highly related 150- to 160-amino acid-long region of sequence designated as the POU domain. Polymerase chain reaction (PCR) amplification of human and rat brain cDNA and rat testes cDNA with degenerate primers to the POU domain led to the discovery of the Brn-1, Brn-2, Brn-3, and Tst-1 POU proteins (240). All of these known mammalian POU proteins have been implicated by *in situ* hybridization as playing a role in the development of the nervous system. A number of other proteins have recently been discovered with homology to the known POU proteins and many more are certain to follow.

The POU domain can be further subdivided into the homeobox-related POU-homeo and the unique POU-specific domains. The POU homeo domain is more highly conserved among the POU proteins than among other homeobox proteins. The homology between Oct-1 and Oct-2 in this region approaches 88%. The POU homeo domain is involved in both DNA binding and in directing the formation of the multiprotein-DNA complex with αTIF. Helix 2 of Oct-1 makes direct contact with αTIF. Thus, substitution of helix 2 of Oct-2 with that of Oct-1 imparts upon Oct-2 the ability to complex with αTIF (661). Conversely, substitution of helix 2 of Oct-1 with that of Oct-2 destroys the ability of Oct-1 to bind αTIF. The POU-specific region is 75 to 83 amino acids long and located in the amino-terminal half of the overall POU domain. The POU-specific domain is 99% conserved between Oct-1 and Oct-2. If the POU-specific domain does play a role in complex formation, it must be at amino acids conserved between Pit-1 and Oct-1 because replacing the POU-specific domain of Oct-1 with that of Pit-1 reduced did not eliminate αTIF complex formation (661). Its function is largely unknown, although in Oct-1 it appears to contribute to DNA binding (673).

The POU-homeo and POU-specific domains are separated by a short nonconserved 15- to 20-amino acid linker. The insertion of an additional six alanines into the linker had no effect on DNA binding, suggesting that the POU domain is a bipartite DNA-binding structure. Such flexibility in the DNA-binding domain might explain the many apparently dissimilar DNA sequences to which Oct-1 appears to bind. Sequences which are identical in only

four of 14 residues have been shown to bind, yet simple point mutations within some binding sites can abolish binding (35).

α-TIF Response Element

As noted earlier in the text, several lines of evidence indicated that the consensus sequence identified by Mackem and Roizman (380,381) [5′ GyATGnTAATGArATT 3′] is the *cis*-site for induction of α genes by αTIF (337). Gaffney et al. (203) designated the *cis*-acting site as the sequence TAATGARAT. For reasons discussed below, the *cis*-acting site is longer and extends upstream.

Gel retardation studies using a 29-bp fragment from the α0 gene (29Rα0) and a 48-bp fragment from the α27 gene (48Rα27) revealed that the *cis*-acting site binds host proteins (334). Both DNase protection and methylation interference studies indicated that those purines whose methylation interfered with the binding of the largest of these host proteins, αH1, was part of the sequence ATGCTAAT, which resembled the consensus binding site ATGCAAAT of several proteins subsequently designated as Oct-1 (333). Extensive mutational analysis of the αTIF consensus site in the context of the viral genome (647,648) led to three conclusions. First, mutagenesis of the ATGCTAAT domain led to a loss of Oct-1 binding and reduced the level of expression of the reporter gene to 6% that of wild-type virus. Second, transversion of the sequence GATATG, which was designed to destroy the consensus octamer binding site but preserve the TAATGARAT sequence also abolished high-affinity binding of Oct-1 and αTIF, but, at vastly increased amounts of probe, trace amounts of bound Oct-1 could be detected, presumably reflecting direct binding of the protein to the TAATGARAT sequence. Consequently, in cells infected with this virus, the reporter gene expression was reduced to 20% to 30% of the level observed in cells infected with the wild-type parent. Further studies showed that expression of the reporter gene correlated with the respective Oct-1 binding levels. Third, mutations in the GARAT domain abolished the formation of αTIF/Oct-1/DNA complexes and reduced the expression of the reporter gene to 20% to 30% of the wild-type level but had no effect on the binding of Oct-1 to the DNA. The stringency of the requirement for a GARAT sequence varied, depending on the cell in which the virus carrying the mutation was tested. In some cell lines, the mutant promoter expressed the reporter gene at a level 80% that of the wild-type. On the basis of these findings, it was postulated that an unknown cellular factor is capable of interacting with Oct-1 to induce gene expression, albeit to a lesser degree than when Oct-1 is complexed with αTIF, that this factor does not require the GARAT sequence, and that certain cell lines possess more of this factor than others. Indeed, such cellular factors have recently been discovered (449).

Stable formation of a complex between purified Oct-1 and αTIF requires an additional factor designated as C1 (212,332,338). Highly purified C1 was reported to consist of a family of polypeptides derived from a common precursor by proteolytic cleavage (303, 332a, 738). C1 binds to αTIF rather than to the Oct-1/DNA complex. An additional factor designated as C2 and forming a complex designated by the same name has also been reported (332,338).

In summary, the multiprotein complex responsible for induction of the α genes is initiated by the Oct-1 binding to the octamer element within the αTIF *cis*-acting site. The αTIF/C1 factor complex binds to the Oct-1/DNA complex and is stabilized by specific interactions between the αTIF and the Oct-1 POU-homeo domain and between αTIF and the GARAT sequences of the *cis*-acting site. Although αTIF interacts directly with the POU-homeo domain, no interaction is detected in the absence of DNA, suggesting that this interaction is of low affinity. Likewise, the DNA-binding affinity of αTIF is extremely low and may require an interaction with Oct-1 *in vivo*. The nature of the interactions of the C2 factor with the complex involve most likely protein-protein interactions as the binding of this component does not affect the DNA footprint of the complex.

Regulatory Functions of α Gene Products

ICP0

The product of the α0 gene is predicted to be 80,000 in molecular weight, but in denaturing polyacrylamide gels it migrates with an apparent molecular weight of 110,000 to 124,000, depending on the type of cross-linking agent used and on the acrylamide concentration. ICP0 oligomerizes in solution (89,174). It has been reported that ICP0 is a virion protein (750), but definitive studies involving separation of "heavy" or complete particles from light particles which may package nonvirion proteins have not been done. The $\alpha 0^-$ mutants are viable in cell culture, and ts mutants have not been reported (585,667). In transient expression systems, ICP0 has been reported to *trans*-activate transfected genes promiscuously by itself or in combination with ICP4 (169–171,208–210,462–464,484,526,589). Of the various experimental designs, the most convincing are those in which the *trans*-activation of target genes was done in conjunction with ICP4.

The transient expression studies suggest ICP0 enhances the function of ICP4, possibly by interacting with it, but this may be only one of its functions. ICP0 response elements have not been identified, although it has been reported that the protein interacts with a cellular protein (426). Deletion mutants in the α0 gene grow in cell culture, albeit more sluggishly than the wild-type virus, particularly at low multiplicities of infection (585,667).

Mutants in ICP0 have been studied in some detail by both Cai and Schaffer (67) and Chen and Silverstein (90).

Overall, it is difficult to argue with the basic conclusion by Chen and Silverstein that, while it is not essential for viral replication in some cells in culture, defects in this gene delay the expression of ß and γ genes and impair viral replication.

ICP4

ICP4 is a phosphoprotein essential for expression of viral genes and for viral replication. Long a subject of study, it has generated a rich, passionate, and at times confusing literature.

Properties of the Protein

This protein is predicted to be 132,835 in translated molecular weight (402). Most limited-passage HSV-1 isolates carry a ts lesion mapping in the α4 gene and 37°C/39°C ratios of plating efficiencies as high as 10^6 have been recorded [e.g., HSV-1(F)]. It is perhaps then not surprising that ts mutants in the α4 gene have been readily isolated by a number of laboratories (361). ICP4 forms three bands designated as 4a, 4b, and 4c in denaturing polyacrylamide gels (483). In cells infected with wild-type virus, the fastest migrating band (4a) has an apparent molecular weight of 160,000 and is readily detected in the cytoplasm after pulse labeling with radioactive precursors (261,262,483,735). It is also the only form accumulating in cells infected with certain α4 ts mutants and incubated at nonpermissive temperatures (320). The other bands (4b and 4c) have apparent molecular weights of 163,000 and 170,000, respectively, and accumulate in the nucleus (337,483). The accumulation of the slower migrating bands coincides with the translocation of the protein into the nucleus and labeling with inorganic ^{32}P added to the medium (183,483). ICP4a and ICP4c can be pulse labeled with ^{32}P during the reproductive cycle long after the synthesis of this protein ceases, suggesting that phosphate cycles during infection (735). As noted above, ICP4 is (ADP-ribosyl)ated (43,506). Adenosyl ribosylation could account for only a fraction of the labeling of ICP4 by inorganic phosphate from the medium (483), inasmuch as phosphate also cycles on and off ICP4, ICP22, and ICP27 (735), but ICP4 appears to be the only α protein to be poly(ADP) ribosylated. Recently, Blaho and Roizman (46) reported that ICP4 is also guanylylated and adenylylated.

ICP4 has been shown to bind to DNA directly in several types of assays (177,335,336,430). Initial studies identified a strong binding site with a consensus sequence of ATCGTCnnnnCnGnn (176). Subsequent studies have identified numerous binding sites which do not correspond to this consensus sequence (335,336,429,430). Studies by Michael and Roizman (428,429) have shown that two molecules of ICP4 bind to both consensus and nonconsensus sites.

The various forms of ICP4 differ with respect to their affinities for the binding sites (430). Papavassiliou et al. (472) reported that, whereas dephosphorylated forms of ICP4 could bind to α promoters, only phosphorylated forms bound to ß and γ gene promoters. Anatomic dissection of the α4 gene in the context of the viral genome has outlined several domains which play a key role in the function of this protein. Most of the key functions of the protein appear to be associated with the carboxyl half of the protein. Mutations in this region affect autoregulation, *trans*-activation of viral genes, intranuclear localization, interaction with DNA, and so forth (137–139,617,634). Wu and Wilcox (746) mapped the ICP4 sequence-specific DNA-binding site to residues 262–490.

To *trans*-activate transcription of HSV genes, ICP4 must interact with a component of the PolII transcriptional complex. Indeed, interaction of ICP4 with TATA binding protein and TFIIB has been reported (632).

ICP4 has been reported to be packaged in virions (749). The uncertainty surrounding this datum rests on the concern that ICP4 detected in these assays may be packaged nonspecifically into "light" particles and is not a bona fide component of the virion. If both ICP4 and ICP0 were to be packaged in virions, we would expect to see transcription of genes in cells infected and maintained in the presence of cycloheximide. This is not the case.

The $\alpha 4^-$ mutants express only α genes, but with time both ß and γ genes are also expressed. To multiply, $\alpha 4^-$ mutants must be grown in cells capable of expressing ICP4 proteins from a copy of the α4 gene embedded in the cellular genome. The observation that ICP4 is required for the expression of genes expressed later in infection has been taken as an indication that ICP4 regulates genes positively. Evidence has also amassed indicating that ICP4 regulates specific α genes negatively. It is convenient to consider these manifestations of ICP4 function separately.

Role of ICP4 in the Expression of α Genes

As noted above, ts mutants in the α4 gene abound. In these viruses, both copies of the gene are mutated, as would be expected for the expression of the ts phenotype (320). The phenotypes of these mutants vary. At the nonpermissive temperature, some mutants express both α proteins and selected sets of proteins normally made later in infection (136,140). A most interesting set of ts mutants in the α4 gene overproduce α proteins at the nonpermissive temperature (136,501,713). There is convincing evidence that ICP4 turns off its own synthesis and that this autoregulation correlates with the binding of the protein to a *cis*-acting site across the transcription initiation site of the gene (138,427,437,542,713). Measurements of α RNAs accumulating in cells infected and maintained at permissive and nonpermissive temperatures indicate that the α genes subjected to repression are primarily α4 and α0 (J. Hubenthal-Voss and B. Roizman, unpublished observations).

In the case of the α0 gene, the binding site ATCGTCactgCcGcc is at position −64 to −49 (335). The α4 gene 5′ untranscribed and transcribed, noncoding domain contains three ICP4 binding sites. Two nonconsensus binding sites map at positions −194 to −171 (site α4-1 distal) and −162 to −145 (α4-1 proximal), whereas one consensus site (α4-2) maps across the cap site (176,335,336,430,447). The function of these binding sites has been the subject of numerous and somewhat contradictory reports.

Roberts at al. (542) reported transient expression assays of the function of the ICP4 binding site at the transcription initiation site of ICP4 (α4-2 site). Deletion of the nucleotides ⁻8 to +30 abolished both binding of DNA and "negative autoregulation." Detailed studies by Resnick et al. (532) showed that ICP4 *trans*-activated a mutated promoter lacking the binding site much more efficiently than the wild-type promoter containing an intact DNA binding site. In contrast, Everett and Orr (173) substituted a mutated promoter lacking the ICP4 binding site for the wild-type promoter in both copies of the viral genome. They reported that the wild-type and mutant viruses did not differ with respect to the accumulation of ICP0 throughout infection. Although the results would support our view that transient expression systems are useful to determine the requirements for expression but not for the regulation of viral genes, in this instance, analyses of the accumulation of mRNA rather than protein would have dealt more directly with the role of the ICP4 binding site on the expression of ICP0. A remarkable feature of HSV gene expression is that the accumulation of many viral proteins is fine tuned at several levels. In the case of ICP0, recent studies indicate that its accumulation is regulated by the products of both U_L13 and α22 (520).

As noted above, ICP4 binds to three sites within the 5′ untranscribed and transcribed, noncoding domains of the α4 gene. The purines whose methylation interferes with the binding of ICP4 to these sites have been mapped (429, 559,560). Mutagenesis of these three sites in a construct in which the 5′ untranscribed and the entire transcribed noncoding domain of the α4 gene were fused to the coding sequence of a reporter gene and inserted into the viral genome revealed the following. First, ICP4 did not bind to the mutated binding sites. Second, identical amounts of RNA of the chimeric gene were recovered from cells infected with viruses carrying wild-type and mutated α4-1 and α4-2 sites linked to the reporter gene and maintained under cycloheximide, indicating that the mutations did not affect either the *trans*-activation of the α4 promoters linked to the reporter gene or the stability of the RNAs. Third, mutations of the α4-2 sites resulted in a seven- to 18-fold increase in the amounts of reporter gene RNA recovered at 4 and 8 hr postinfection. Fourth, mutations of either the distal or proximal α4-1 binding sites alone or in combination did not affect the recovery of reporter gene RNA at these times postinfection, whereas the mutagenesis of all (α4-1 and α4-2) binding sites increased at least threefold the amount of RNA recovered compared to mutagenesis of the α4-2 site alone (427). These results strongly support the hypothesis that (i) ICP4 acts by binding to both consensus and nonconsensus sites on DNA, (ii) autoregulation of α4 gene expression is the consequence of binding of ICP4 to DNA, and (iii) the ICP4 binding sites upstream from the cap site can contribute to the repression of gene expression.

Koop et al. (325) inserted wild-type and mutated ICP4 binding sites into a variety of promoter domains. They noted that ICP4 binding sites upstream or downstream of TATA boxes effectively decreased gene expression from these promoters. An interesting finding was that DNA synthesis alleviated the apparent repression caused by the insertion of the wild-type ICP4 binding sites.

Role of ICP4 in the Expression of ß and γ Genes

ICP4-dependent activation of transcription of a ß gene embedded in the viral genome occurs from a much lower level of basal expression than that seen from an isolated gene introduced in cells and selected for *tk* expression. After *trans*-activation with ICP4, the level of *tk* gene expression is higher than that attained in cells transfected with the isolated *tk* gene but not as high as that seen in lytically infected cells. ICP4 DNA binding sites in the domain of the *tk* gene both upstream of the cap site and downstream from nucleotide +50 have been demonstrated by several groups (336,470–472). Studies by Halpern and Smiley (231) and by Mavromara-Nazos and Roizman (396) have failed to demonstrate a significant role of the binding to sites downstream of nucleotide +51. The report by Papavassiliou et al. (472) that the binding of ICP4 to ß and γ genes requires infected cell factors and is determined by both concentration and phosphorylation of ICP4 is consistent with and supports the conclusion that ICP4 interacts with cellular factors and both enhances and depends on them for the stability of its own binding to DNA.

In infected cells, ICP4 is required but is not sufficient for efficient and timely expression of γ genes, particularly γ_2 genes. DNA binding sites have been observed in both 5′ untranscribed and transcribed, noncoding domains of genes (428,430,684). The role of these sites is unclear.

Does ICP4 block ß and γ gene transcription, *trans*-activate it, or both? The evidence that, in cell-free systems, ICP4 increased the transcription of gD, a γ_1 gene (685), is not in itself impressive since (a) the ICP4 binding site tested was upstream from the reported minimal sequence required by gD to be regulated as a γ_1 gene (nucleotide −55) (148,150); (b) in transient expression systems, for what it is worth, late γ_2 gene expression was activated at low concentrations of the ICP4 gene but not by high concentrations of the gene; and (c) the amount of ICP4 used in that study was arbitrary, without relevance to either known positive or negative *trans*-activation. Subsequently, Tedder et al. (684) reported that ICP4 binding sites enhance the tran-

scription of the gD gene *in vitro.* However, studies involving mutagenesis of the ICP4 binding sites within the domains of the gD gene failed to affect the transcription of that gene (631).

In other studies, some of the characteristics of γ_2 gene regulation, i.e., expression of the gene late in infection and sensitivity to inhibitory effects of PAA, were transferred to the *tk* gene by substitution of the *tk* cap site and a portion of the 5′ transcribed noncoding domain with the corresponding sequences of the U_L49.5 gene. The substituted γ_2 sequences contained two ICP4 binding sites; mutagenesis of one of these sites led to a loss in the capacity of the chimeric gene to be expressed late in infection (325). While the hypothesis that ICP4 does not act directly on the DNA cannot be dismissed out of hand, it seems likely that ICP4 acts in at least two ways, by stabilizing the assembly of TATA box-dependent transcriptional factors and by a local effect at the site of binding. Studies showing that ICP4 bends DNA have been reported (175). The jury evaluating the role of ICP4 binding sites in ß and γ genes is still out. While the data clearly indicate that functional ICP4 is required for the expression of these genes, the precise mechanism by which ICP4 acts remains elusive.

Much has been made of the observation that α4 and the equivalent gene product of pseudorabies virus induce not only herpesvirus genes but also adenovirus and cellular genes (e.g., ß globin), which are introduced into cells by transfection (41,180,228). More recent studies have shown that the *trans*-activation of adenovirus late gene expression by the ICP4 equivalent of pseudorabies virus is through enhanced formation of transcription initiation complexes (1). Although these studies are consistent with the hypothesis that ICP4 may stabilize specific sets of transcriptional factors on the DNA, they do not exclude the possibility that weak interactions with DNA may be necessary for *trans*-activation of gene expression.

ICP27

The realization that this nuclear phosphoprotein performs a plethora of diverse regulatory functions throughout infection emerged only in recent years (588). The key functions and properties of ICP27 may be summarized as follows.

1. ICP27 is an essential gene. Null mutants do not replicate; they exhibit a gross decrease in viral DNA synthesis and late gene expression. Some ts and nonsense mutants do not block viral DNA synthesis but do decrease the expression of late genes (419,584).
2. The key phenotype of ts mutants studied in some detail indicate that ICP27 exhibits both activator and repressor functions. Thus, in the absence of functional ICP27, α genes are overexpressed, whereas late genes are poorly expressed (398,533,584,635). As articulated by McCarthy et al. (398), the general conclusion is that ICP27 acts at a transcriptional level.

   More recent studies indicate that ICP27 does have a role in transcriptional regulation at two levels. First, ICP27 determines which polyadenylation signal is used where more than one signal is present (418). Second, it has been noted that the amount of spliced RNA is decreased in the presence of functional ICP27. Splicing studies support the hypothesis that ICP27 directly inhibits splicing by sequestering snRNPs (236).
3. Studies on ICP27 mutants have identified both repressor and activator domains which map in the carboxy-terminal half of the protein (235,419,433,534). Amino-terminal domains have been shown to be essential for optimal levels of DNA synthesis and for localization of ICP27 in the nucleus. One domain localized roughly between amino acids 138 and 152 bears a striking similarity to several cellular proteins which have been implicated in nuclear RNA processing and appears to be required for nucleolar localization (347).
4. ICP27 binds to single-stranded DNA-agarose columns and is eluted by high salt. In these studies, ICP27 copurified with an unidentified protein 110,000 in molecular weight. In addition, the predicted amino acid sequence has a potential metal binding domain, and *in vitro* the protein binds zinc. This domain is located at the carboxyl-terminal 105 amino acids. Conservation of this domain is essential for at least some of its regulatory effects (702).

In summary, ICP27 has been reported to perform an extraordinary range of regulatory feats which include selection of transcriptional termination sites, inhibition of RNA splicing, stimulation of DNA synthesis, and posttranscriptional destabilization of α mRNA (588). This is a remarkable range. In time, it may decrease as some of the manifestations now thought to be due to direct action of the protein are recognized to be the consequences of secondary events in a regulatory cascade.

ICP22

Inasmuch as this gene is dispensable (499), it has not been studied intensively. The studies done to date (603) indicated the following. The $\alpha22^-$ virus multiplied in Vero and HEp-2 cells as efficiently as the parent virus. In BHK and RAT-1 cell lines and in human embryonic lung cells, the plating efficiency of $\alpha22^-$ virus was reduced, and the yield was multiplicity dependent. Moreover, in these cells, the shut off of synthesis of ß proteins was delayed, and the expression of γ proteins and the number of capsids detected in infected cells was reduced. Deletion of α22 had no effect on viral DNA synthesis. The α22 protein is phosphorylated by several kinases, including U_L13 and U_S3 (520,521) and is adenylylated by protein kinase II *in vitro* (432). The phenotype of $\alpha22^-$ cannot be differentiated from that of U_L13^- in cells in culture (520).

ICP47

This gene is dispensable (377,395) and discussed in more detail in the section on viral modulation of host response to infection.

Other Regulatory Proteins

Recently, it was noted that U_S11, an abundant γ_2 protein, has two functions of potential importance in regulating gene expression (573,574). First, U_S11 protein was found to accumulate in nucleoli, and subsequent studies have shown that it binds to the 60S ribosomal component late in infection, in newly infected cells, and in cells constitutively expressing the protein (573). Second, U_S11 protein binds specifically in a conformation-dependent manner to the mRNA specified by the U_L34 open reading frame (574). Cells infected with a mutant deleted in the U_S11 gene accumulated a truncated form of the U_L34 mRNA. The truncated mRNA is not polyadenylated, but it is transported into the cytoplasm. The U_S11 protein binds to the truncated U_L34 mRNA at or near its terminus. The nucleotide sequence of the 3′ terminus of the truncated mRNA suggests that the truncation takes place immediately before a stem-loop structure. U_S11 protein may act as an antiterminator of transcription of the gene. Inasmuch as no cell line has so far exhibited a phenotype associated with the truncation, the function of the U_S11 protein in regulating U_L34 mRNA accumulation is not known.

Regulation of α Gene Expression

The induction of α genes by αTIF and the autorepression of the α4 gene by its product have been discussed in detail earlier in the text. Although the focus of much of the current studies on α gene regulation centers on these two phenomena, it seems important to stress several observations.

First, from the initial studies of α gene-chimeras (498), it has been observed that α genes are efficiently expressed in transfected cells or in cell lines carrying genes under α promoters. These studies indicate that α gene promoters contain *cis*-acting sites for other transcriptional factors in addition to the αTIF-specific response element. In fact, the studies by Spector et al. (647,648) indicate that cells vary in their capacity to express α genes in the absence of functional αTIF *cis*-acting elements.

The second issue concerns the turn off of synthesis of α proteins. Although the studies by Ackermann et al. (4) led to the conclusion that at least some of the α proteins continue to accumulate throughout infection, there is a significant decrease in α protein synthesis with the onset of synthesis of ß and γ proteins. However, late in infection, Oct-1 is still present, and there is significant accumulation of αTIF—a condition which should result in stimulation rather than shut off of α protein synthesis. Attempts to demonstrate alteration in Oct-1 (e.g., phosphorylation) late in infection have not been productive (F. C. Purves, D. Spector, and B. Roizman, unpublished data). One possible explanation is that the association of VHS with αTIF cited earlier in the text precludes the function of αTIF as a *trans*-activator.

The last point that should be made concerning α gene regulation is that the accumulation of some α proteins may be fine tuned at a posttranscriptional level. The observation that ICP0 accumulation in in HEp-2 and especially in BHK cells was affected by the U_L13 protein kinase raises the possibility that the actual amount of α proteins present at any time may be regulated at many different levels by numerous cellular and viral factors.

Regulation of ß Gene Expression

Exemplified by the *tk* gene, ß genes appear to have the capacity to be expressed in the context of the cellular genome in the absence of other viral gene products. There is a general agreement that the response elements required for the expression of ß genes consist of binding sites for cellular transcriptional factors, a TATA box, and a cap site (413,415), although removal of all known response elements does not appear to ablate entirely ß gene regulation (273,274). In the context of the viral genome introduced by infection into permissive cells, these genes require the expression of α genes and especially of functional ICP4. The level of expression of the *tk* gene in the presence of functional α proteins is higher than that seen in cells stably expressing the *tk* gene in the absence of other viral genes (337).

It would seem that *trans*-activation of ß genes in the context of the viral genome involves two functions, i.e., (i) release from a repressive state and (ii) *trans*-activation. Since neither occurs at the nonpermissive temperature in cells infected with ts mutants in the α4 gene, then at least one, the initial event, depends on ICP4. To aficionados of transient expression, it is worthwhile to point out that, in cells transfected and selected for *tk* activity, the ratio of induced to basal enzyme activity after *trans*-activation with virus is considerably lower than that obtained in cells transfected with a plasmid containing the *tk* gene and another marker and selected for the other, covalently linked marker (337). We interpret this to indicate that, for a constant ratio of *tk* genes per cell, the fraction of derepressed *tk* genes is higher in the cells selected for the TK enzyme activity but that α4 derepresses *tk* genes in both systems to about the same level.

There is little doubt that *trans*-activation of ß genes is enabled by ICP4. The wording of this statement itself betrays how little we know of the precise mechanism by which this takes place.

Regulation of γ Gene Expression

The key questions regarding γ gene regulation are why are the genes not expressed early in infection, what is the nature of *trans*-activators, and what is the identity of the *cis*-acting sites? The mechanism by which HSV regulates its late gene expression remains a formidable challenge and, as such, deserves special consideration. While the exact mechanism for γ gene regulation has yet to be elucidated, there is evidence for (a) involvement of α proteins, (b) inhibition of expression early in infection, (c) response elements in both 5′ transcribed noncoding and coding domains of γ genes, and (d) activation of gene expression after the onset of viral DNA synthesis.

Environmental Constraints on γ Genes for Proper Expression

Initial studies on HSV-1 γ genes were done in transient expression systems transfected with chimeric genes consisting of a reporter gene under the control of a γ gene promoter. In these studies [e.g., those of Dennis and Smiley (141) on γ_1VP5 or those of Silver and Roizman (625) on γ_2UL49.5 promoters], the expression of the γ-*tk* gene transfected into TK-negative cells was low compared to that of the *tk* under its natural promoter. The expression was increased by infection of the cells with a tk^- mutant. Silver and Roizman (625) found that, when cells were treated with the inhibitor of viral DNA synthesis, PAA, and infected with a tk^- mutant, the cells still produced high levels of TK activity. Thus the *tk* gene was regulated as a ß gene rather than as a γ gene. Similar results were obtained with cell lines that contain a stably integrated gC, a γ_2 gene (14). In the environment of the cellular genome, the gene was not expressed unless the cells were infected with HSV. In addition, gC was produced in cells infected with a ts DNA^- virus at the nonpermissive temperature or infected with wild-type virus and treated with PAA, indicating that it was regulated as a ß gene. These observations led to the conclusion that γ genes must be studied within the context of a viral genome in productive infection.

Dependence of γ Gene Expression on Viral DNA Synthesis

Two key hypotheses could explain the dependence of γ gene expression on DNA synthesis. The first is that γ genes are negatively regulated during the early phase of viral infection either (i) by the binding of *trans*-acting negative factors or (ii) by constraints placed on the late genes by a particular DNA secondary structure formed in the vicinity of the γ genes. Viral DNA replication could, in either case, relieve the block and allow full expression of γ genes. The second hypothesis predicts the production or modification of a *trans*-acting factor during viral DNA replication that allows the activation of γ gene expression.

To differentiate between these two hypotheses, cells infected for 6 hr with a tk^- mutant were placed in a medium containing PAA and superinfected with a virus containing its *tk* gene under the control of the promoter of the $\gamma_2$49.5 gene. Assays done on the doubly infected cells failed to detect the expression of the *tk* gene of the superinfecting virus and indicated that the expression of γ genes is tightly linked to viral DNA synthesis and the effect of DNA synthesis is mediated by a *cis*-acting function (397).

It has been reported that ICP8, the HSV-1 single-stranded DNA binding protein essential for viral DNA synthesis, may be a negative regulator of γ gene expression. In addition, ICP8 is an essential protein for viral DNA synthesis inasmuch as the mutant HSV-1 KOS1.1 *ts*18, at the nonpermissive temperature, blocked viral DNA synthesis but allowed low level expression of the γ_2 gC gene even in the presence of inhibitors of DNA synthesis (218). Conversely, certain mutants in the α27 gene downregulate γ gene expression in the face of DNA synthesis, as does an $\alpha22^-$ mutant in restrictive cells. Viral DNA synthesis is necessary but not sufficient for the proper expression of γ genes.

Role of α and ß Gene Products in γ Gene Regulation

The roles of α and ß genes in the expression of γ and, particularly, γ_2 genes may be summarized as follows. First, functional ICP4 is required but is not sufficient for the expression of γ genes. Second, in most instances tested, ICP0 enhances the capacity of ICP4 to *trans*-activate γ genes. The hypothesis that α0 regulates γ gene expression by regulating α27 which in turn affects the switch from ß to γ gene expression is interesting, but much more data will be needed to support it (90). Third, deletion of the ICP27 gene resulted in the downregulation of the expression of γ genes. In the case of specific mutants in this gene, γ gene expression was reduced, notwithstanding viral DNA synthesis (235,398,419,533,534,635). Fourth, the requirement for ICP22 for optimal gene expression is cell-type dependent (603). As noted above, ICP22 is posttranslationally modified by phosphorylation by U_L13 (521). In the absence of U_L13, γ_2 gene expression is phenotypically similar to that seen in cells infected with U_L13^- virus (520). Fifth, other than the ß genes required for viral DNA synthesis and the protein kinase described above, several others exert a profound effect on the expression of γ genes.

Cis-Acting Sites Involved in γ Gene Regulation

There is an incipient convergence of views on the structure of γ gene promoters and, particularly, of γ_2 promoters. One series of studies suggested that they consist simply of a specific TATA box (188,257,294). Indeed, Johnson and Everett (294) reported that the cap site and TATA box of the gene encoding U_S11 is all that is required for "fully efficient regulated activity." At the other extreme, Mavro-

mara-Nazos and Roizman (396) demonstrated that a reporter gene driven by the 5′ nontranscribed sequences of the *tk* (ß) gene fused to the 5′ transcribed noncoding domain of the U_L49.5, a γ_2 gene, was regulated as a γ_1 gene, leading to the hypothesis that response elements of γ_2 genes are located in the 5′ transcribed noncoding domains and that fusion of the ß 5′ untranscribed domains to the γ_2 response elements yields promoters with characteristics of γ_1 genes. Similar results were obtained with a chimeric promoter consisting of the *tk* 5′ untranscribed region fused to the gC (a γ_2 gene) 5′ transcribed noncoding domain (258, 396,722). Homa et al. (258) concluded that the γ_2 response elements resided in the 5′ transcribed noncoding domains. The situation may be far more complex. Thus the U_L24, a γ gene, was expressed when its TATA box was replaced by that of the gC (γ_2) gene but not when it was replaced by the TATA box of the ß-*tk* gene or that of the $\gamma_2 U_S$11 gene (308). Linker-scanning mutations in the gC and gH promoters led to the conclusion that the genomic sites important for the expression of γ_2 genes are the TATA box, the cap site, and nucleotides +30 to +40 relative to the cap site (656). The mutations in the TATA box and cap site affected transcription, whereas the mutation in the 5′ transcribed noncoding domain had little effect on transcription but had a dramatic effect on the amount of reporter protein produced. Although all three mutations reduced the expression of the reporter gene, none altered its temporal regulation.

Clues to the possible function of the γ_2 5′ transcribed noncoding domains emerged from studies of a series of chimeric genes consisting of the gC (γ_2) 5′ untranscribed and transcribed noncoding domains fused to the chick ovalbumin coding sequences and inserted into the viral genome. The key observation is that some genes with deletions within the 5′ transcribed noncoding domain were expressed in the presence of cycloheximide, although the indicator gene was expressed to a higher level late in infection (R. King, M. Arsenakis, A. Poon, and B. Roizman, unpublished data).

At least two hypotheses could explain the observation that deletion mutants in the 5′ transcribed noncoding sequences of a γ_2 gene could be expressed as an α gene. First, the 5′ noncoding domain may contain a site that allows the binding of specific *trans*-acting proteins necessary for the regulation of γ_2 gene expression. A survey of several γ_2 genes of the published HSV-1 DNA sequence (402) failed to reveal a potential response element conserved in all or most γ_2 genes, although the data do not exclude the possibility that the shared response element is highly degenerate. Second, the 5′ transcribed noncoding domain may form a secondary structure which affects expression of the γ_2 genes. Specifically, analyses of the nucleotide sequences with the RNAFOLD program (Genetics Computer Group, Madison, WI) led to the conclusion that the γ_2 5′ transcribed noncoding domains could form long and apparently stable stem-loop structures, although evidence that they actually exist is lacking. Whereas the wild-type sequence is potentially capable of forming a stem 25 bp long, the mutation which confers upon the chimeric gene the capacity to be expressed as an α gene lost the ability to form a stable stem-loop structure (R. King, M. Arsenakis, A. Poon, and B. Roizman, unpublished data).

Irrespective of whether the secondary structure theory is supported by future studies, the available data suggest that the 5′ transcribed noncoding domains contain response elements for a repressor which precludes expression of γ_2 genes under conditions which enable the expression of α and ß genes and an activator which enables their expression in the absence of the repressor (R. King, M. Arsenakis, A. Poon and B. Roizman, unpublished data) (397). It is tempting to speculate that the function of DNA synthesis and of ICP8 is to disable the repressor and that the function of ICP27 relates to the function of the *trans*-activator. The available data also suggest that the repressor is absent or inactive in γ genes embedded in the context of nonviral episomes or cellular chromosomes.

Posttranscriptional Regulation

The evidence for posttranscriptional controls is based on reports that translocation of viral transcripts into the cytoplasm appears to be regulated (298,300,329). Specifically, the genetic complexity of the RNA accumulating in the nuclei of cells infected with HSV in the presence of cycloheximide and maintained in medium containing the drug was greater than that observed in the cytoplasm. In retrospect, the interpretation of the data is not clear. The failure to demonstrate RNA complementary to ß genes (e.g., to the *tk* gene) in nuclei of infected cells treated with cycloheximide (361) suggests that the transcripts accumulating in the nuclei might be random transcripts of the DNA rather than transcripts of specific genes belonging to the ß and γ groups.

The evidence for translational regulation is based on several observations. Specifically, the inhibition of host protein synthesis by structural components of the virion soon after infection (184,458,530) and the inhibition of α gene product synthesis by subsequent gene expression (183,262) are translational events, inasmuch as they occur in physically and chemically enucleated cells. A significant finding to emerge from the studies by Read and Frenkel (530) is that virion structural components exert an inhibitory effect on both host and α protein synthesis, inasmuch as mutants defective in the virion host shut-off function produce more α gene products than their wild-type parents.

Lastly, several studies reported here have ascribed posttranscriptional regulatory functions to ICP27 and to the two protein kinases encoded by U_L13 and U_S3, respectively.

HSV Gene Regulation: The Problem in Experimental Designs

The "gold standard" for the studies of viral gene regulation is the pattern of expression in productive infection

of natural or reporter genes contained in the viral genome. Tests of modified *trans*- or *cis*-acting domains of individual genes are easier to perform and may, in some cases, be more meaningful if they can be done in the environment of the cell and in the presence of only a minimal amount of viral genetic information. However, the validity of such tests hinges on the extent to which they reproduce the regulation of the gene embedded in the viral genome and expressed in the course of viral infection. The expression of isolated α genes, in biochemically transformed cells or in transient expression systems, appears to mimic to some extent the regulation of the corresponding genes contained in viral genomes during productive infections (498). Notwithstanding the massive number of transfections which argue that ICP0 is a promiscuous *trans*-activator, supporting evidence from studies on deletion mutants in the α0 gene is not readily available. The transfection system apparently fails if more than two components of the regulatory pathway are introduced into the cell simultaneously, for example, the cotransfection of αTIF, α4, and the intended target gene of α4 (542). In the case of γ_2 genes, the transient expression system yields totally false results; viral genes permanently integrated in cellular genomes or transiently expressed after transfection are regulated as ß genes (14,47,625). The transfection system has given rise to a veritable cottage industry, but the data generated by it are not totally reliable. What is the evidence that viral genes other than those carrying the α *cis*-acting sites can be regulated in that system in a mode which resembles viral gene regulation? If γ_2 genes are regulated as ß genes, what is then the evidence that ß genes in transfected cells are regulated as bona fide ß genes?

FATE OF THE INFECTED CELL

Cells productively infected with herpesviruses do not survive. Almost from the beginning of the reproductive cycle, the infected cells undergo major structural and biochemical alterations that ultimately result in their destruction.

Structural Alterations

Changes in Host Chromatin

As described in detail elsewhere (557) and shown in Fig. 4, one of the earliest manifestations of productive infection is in the nucleolus; it becomes enlarged, displaced toward the nuclear membrane, and ultimately disaggregates or fragments. Concurrently, host chromosomes become marginated, and later in infection the nucleus becomes distorted and multilobed. The numerous protrusions and distortions have in the past been mistaken for amitotic division (302,602). Margination of the chromosomes may or may not be linked with the chromosome breakage reported by numerous investigators (557).

Virus-Induced Alteration of Cellular Membranes

Duplication and Folding of Intracellular Membranes

Changes in the appearance of cellular membranes and, in particular, of nuclear membranes is characteristic of cells late in infection. Deposition of material (tegument proteins?) on the inner surface facing the nucleoplasm or cytoplasm, but not in the space between inner and outer lamella or cisternae of the endoplasmic reticulum, results in the formation of thickened patches along the membranes. Ultimately, the patches in the nuclear membrane coalesce and fold upon themselves to give the impression of reduplicated membranes (Fig. 6) (111,166,356,401,439,454,500, 601,624,743).

Insertion of Viral Proteins into Cellular Membranes

The first inkling that herpesviruses modify cellular membranes was based on the observations that mutants differ from wild-type strains with respect to their effects on cells; while wild-type viruses usually cause cells to round up and clump together, some mutants cause cells to fuse into polykaryocytes (158,552). These observations led to the prediction that herpesviruses alter the structure and antigenicity of cellular membranes—a prediction fulfilled by the demonstration of altered structure and antigenic specificity (541,564,563,569) and the presence of viral glycoproteins in the cytoplasmic and plasma membranes of infected cells (243,567,568,642).

Polykaryocytosis

Both HSV-1 and HSV-2 cause infected cells to round up and cling to each other. Some viral mutants cause cells to fuse into polykaryocytes; this fusion may be cell type specific or cell type independent (158,252,582). Polykaryocytosis has been studied for several reasons: as a probe in the structure and function of cellular membranes, reflected in the "social behavior of cells;" as a tool for analyses of the functions of viral membrane proteins; and as a model of the initial interaction between HSV and susceptible cells that results in the fusion of the viral envelope and the plasma membrane (74,552,639,640). Cell fusion induced by HSV also requires conditions which favor processing of high-mannose glycans to complex glycans, but in this instance it is not clear whether complex glycans must be present only in viral glycoproteins present on the surface of the infected recruiter cells or on both the recruiter cells and on the uninfected cells to be recruited into polykaryocytes (74,639,640).

Polykaryocytosis can be viewed as an aberrant manifestation of the interaction of altered membrane domains of infected cells and unaltered membranes of juxtaposed cells (552). Genetic analyses have shown that mutations

(*syn*) which confer the capacity to fuse cells map in at least five and possibly more loci within the viral genome (22,54, 132,367,386,491,493,576,582,748). These loci map within the domains of gB, gK, gL, U_L24, and U_L20. One interpretation of this observation is that the membrane proteins form complexes whose structure and conformation become altered by mutations in any of the component polypeptides and that the changes in the conformation are similar to those which occur in the envelopes of virions interacting with the plasma membrane (582).

Host Macromolecular Metabolism

Background

A characteristic of herpesvirus-infected cells is the rapid shut off of host macromolecular metabolism early in infection. Thus, host DNA synthesis is shut off (562), host protein synthesis declines very rapidly (554,674,676), and glycosylation of host proteins ceases (642).

Herpes simplex virus-induced host shut off occurs in two stages. The first stage, documented initially by Fenwick and Walker (184) and by Nishioka and Silverstein (456–458) involves structural proteins of the virus and does not require *de novo* protein synthesis. Thus, HSV shuts off host protein synthesis in physically or in chemically enucleated cells (183); the shut off was effected by density gradient-purified virus but not by purified virus inactivated by heating or neutralization with antibody. The shut off is faster and more effective in HSV-2- than in HSV-1-infected cells; this observation permitted the initial mapping of the genetic locus that confers upon HSV-1 × HSV-2 recombinants the accelerated shut off characteristic of HSV-2 (181). Isolation of *vhs* mutants which fail to shut off host polypeptide synthesis in HSV-infected cells (530) has demonstrated more conclusively that this function is due to a virally encoded protein.

The second stage requires *de novo* synthesis of proteins after infection (183,261,262,456,457,627). The shut off coincides with the onset of synthesis of ß proteins, but the experimental results do not exclude the possibility that the shut off is caused by γ rather than ß gene products.

Expression of the vhs *Gene and the Function of Its Product*

The *vhs* function was initially mapped to 0.52 to 0.59 by (181). Isolation of a mutant defective in this function (530) allowed further mapping of the gene responsible. Mapping studies by Oroskar and Read (469) and Kwong et al. (342) have identified sequences mapping from 0.604 to 0.606 on the viral genome (U_L41 open reading frame) as being responsible for the *vhs*$^-$ phenotype of the mutants. U_L41 RNA is expressed as a γ_1 gene (200). The products of U_L41 are an abundant phosphoprotein of M_r 58,000 and a less abundant, more extensively phosphorylated protein of apparent M_r of 59,500. Only the faster migrating protein is found in virions (531). It has been reported that the $U_2$41 protein forms a complex with VP16 (630).

Early studies showed that virion components were responsible for destabilization and degradation of host mRNA (184). Further studies have led to the conclusion that the virion component responsible for both mRNA destabilization and degradation, *vhs*, is also responsible for a nondiscriminatory destabilization and degradation of viral α, ß, and γ mRNAs (182,342,469,595,670).

In cells infected with the *vhs*$^-$ mutant, host protein synthesis is not shut off at least early in infection; α and ß protein synthesis are somewhat prolonged compared to wild type. Both of these effects have been shown to be due to a stabilization of host and viral mRNAs; in cells infected by *vhs*$^-$ mutants, mRNAs are not degraded as rapidly as in cells infected by wild-type virus.

This function confers at least two advantages on the virus. First, it removes preexisting host mRNA from the pool of translatable messages, allowing the viral mRNAs to take over the pool rapidly. Second, destabilization of viral mRNAs allows a rapid transition from one regulatory class to the next. In the absence of the *vhs* function, α and ß proteins are produced beyond the time spans normally seen; the positive transcriptional controls discussed in the rest of this paper are not enough to ensure efficient α to ß and ß to γ transitions. Although the *vhs*$^-$ mutation is not lethal, wild-type virus does have a growth advantage in tissue culture, indicating that efficient separation of the regulatory classes is helpful to the virus.

VIRULENCE

Virulence, if defined as the ability of the virus to cause disease, is composed of several parts. During infection of humans, viral disease includes primary and recurrent epithelial lesions as well as disseminated disease and encephalitis. In studies on the molecular basis of disease induced by HSV, the end point of the research objective—the disease—is often taken to be synonymous with the destruction of central nervous system (CNS) tissue. In healthy nonimmunocompromised humans, encephalitis occurs rarely but with catastrophic results (727). In experimental animals, it is frequently a major component of the disease. Neurogrowth, as measured solely by intracerebral inoculation of virus, is the most commonly measured aspect of virulence. Direct injection of virus into the CNS measures the capacity of the virus to destroy an amount of CNS tissue that will result in death before the immune system blocks further virus spread. Because in most instances destruction of the CNS and death are related to virus multiplication, in quantitative terms, the growth of the virus in the CNS is measured in terms of the amount of virus required to reach a specific level of mortality (50% of inoculated animals).

A second attribute of virulence is invasiveness—the capacity to reach a target organ from the portal of entry. To disseminate to the target organ, it may be necessary for the virus to multiply at peripheral sites. In experimental systems, virulence is composed of (a) peripheral multiplication, (b) invasion of the CNS, and (c) growth in the CNS. Peripheral growth and invasiveness into the CNS can be quantified by measuring the amount of virus recovered at the peripheral site and in the CNS as a function of the quantity of virus inoculated at a peripheral site, i.e. footpad, eye, ear, and so forth.

Several types of studies have been done to define genes required for neurovirulence or neuroinvasiveness. In the first, the doses lethal to 50% of inoculated animals (LD_{50} values) of wild-type isolates taken either from CNS tissues of encephalitic patients or from facial or genital isolates of the same patients or from normal individuals have been compared in mice inoculated by intracerebral or peripheral routes. Although the encephalitis isolates appear to have a slightly lower average LD_{50} as compared to nonencephalitis strains, no correlation was apparent between encephalitis in humans and neurogrowth or neuroinvasiveness in experimental animals (R. J. Whitley, personal communication). However, as a variant of this approach, infection of neurons in culture has indicated that virus isolates from encephalitis cases are better able to infect and be transported to the cell body than isolates from peripheral lesions (40).

Numerous studies have been undertaken to assess the relative abilities of various viral mutants to grow in the CNS after direct intracerebral inoculation. What has emerged from the accumulated data is that so-called neurovirulence factors are not the exception, but the rule. In almost all virus mutants tested to date, including a number of HSV-1 × HSV-2 recombinants, many ts viruses, a variety of spontaneous mutants, and a myriad of recombinant viruses with deletions in one or more genes, LD_{50} values following intracerebral inoculation ranged from 100- to 100,000-fold higher than those of the parental strains. Experiments utilizing nongenetically engineered viruses mapped loci implicated in "neurovirulence" to a number of regions, including the *tk* gene and sequences around the right terminus of the L component (82,185,282–284,322, 511,577,652,689,690). Recombinant viruses with elevated intracerebral LD_{50} values include those with deletions in the genes encoding ICP22, U_S2, U_S3 protein kinase, U_L13, U_L16, U_L24, gG, gJ, gE, gI, ribonucleotide reductase, TK, $\gamma_1 34.5$, U_L55 and U_L56 (E. Kern, B. Meignier, J. Baines, R. J. Whitley, A. Sears, and B. Roizman, unpublished results) (68,421,422,603,718,728). Most of the attenuated deletion mutants listed above also exhibit impaired growth in peripheral tissues (e.g., cornea), as assayed either by the occurrence of epithelial lesions or by quantitation of infectious virus in the tissues, indicating that the genes deleted are required generally for growth in differentiated and/or polarized cells, not specifically for growth in neurons or in CNS tissues (A. Sears, B. Meignier, and B. Roizman, unpublished results) (57,279,280). In fact, the only genes that have, to date, been shown not to be required for neurovirulence in the murine model are gC, U_S9, U_S10, U_S11, and $\alpha 47$ (R. J. Roller and B. Roizman unpublished data) (459). Since the function of $\alpha 47$ appears to be human cell specific (754), it is conceivable that, if the U_S9-11 gene cluster shares in this property, their function will be inapparent in the mouse.

The animal models have thus so far failed to identify any viral function uniquely required for neurogrowth.

LATENCY

The ability of HSV to remain latent in the human host for its lifetime is the unique and intellectually most challenging aspect of its biology. The virus enters sensory nerves innervating the cells of mucosal membranes (Fig. 12). In latently infected neurons, the viral genome acquires the characteristics of endless or circular DNA (195,424,543, 544). To our knowledge, the virus expresses no functions which are required for the establishment or maintenance of the latent state. In a fraction of neurons harboring latent HSV, the virus is periodically reactivated; infectious virus is carried back to peripheral tissues by axonal transport (110), usually to cells at or near the site of initial infection (81,122,222,551). Depending on the host immune response, the resulting lesion may vary considerably in severity, from barely visible vesicles to rather severe, debilitating lesions in immunosuppressed individuals. The clinical aspects of latent infection and reactivation are discussed in Chapter 73. This section concerns the molecular biology of latency.

HSV Latency in Experimental Systems

Experimental Models

The most useful model systems are mice, guinea pigs, and rabbits. In the mouse, latent infection is readily established after eye, footpad, or ear inoculation, but the rate of spontaneous reactivation is extremely low (50,51,248,249, 662). Latent virus in the rabbit does reactivate spontaneously (450). The guinea pig shows recurrent lesions after vaginal infection with high doses of HSV-2 (651).

At the other extreme are latency models in cells cultured *in vitro*. Neurons are nonpermissive at the time they harbor the virus in the latent state, and in the ganglia their permissivity is transient. When placed in culture, neurons become permissive. Those that contain latent virus activate its multiplication. It has been reported that neurons retain virus in a latent state in the presence of nerve growth factor and, conversely, that the virus is activated when the growth factor is withdrawn (734). In this system, only latency-associated transcripts (LAT) are detectable (146). A protein encoded by an open reading frame within the stable LAT intron has been reported (147), but the detected

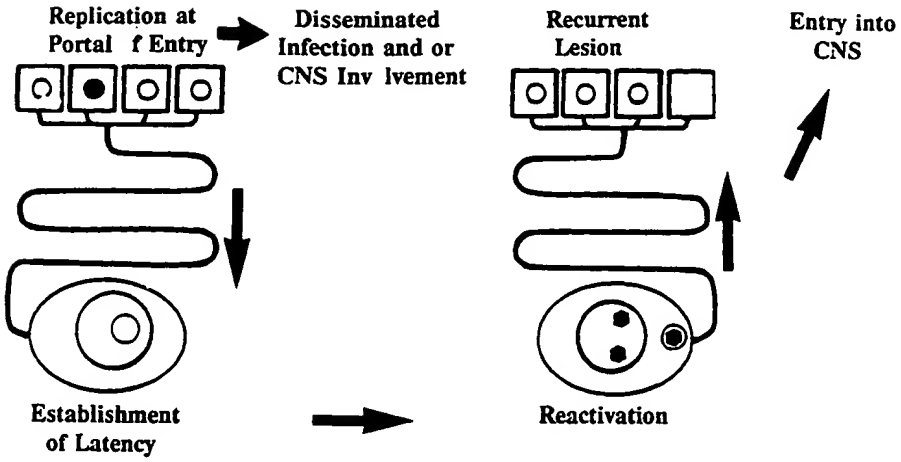

FIG. 12. Natural course of HSV infection *in vivo*. Virus first replicates in epithelial cells (*squares*) at the portal of entry and then moves through neurites (*curved lines*) to establish latent infections in neurons (*ovals*).

protein is several times the predicted size, and the report has never been confirmed. Attempts to detect proteins encoded by several of the major open reading frames 3′ of the $\gamma_1$34.5 gene but within the domain of the 8.3-kb LAT have been unsuccessful (346).

A number of laboratories have reported the maintenance of the viral genome by rendering cells nonpermissive by a variety of methods (106,107,578,729–733). To our jaundiced eye, the state of nonpermissivity induced in cells in culture by elevated temperatures, interferon, or antiviral drugs is not equivalent to the nonpermissive state of the neuronal cells *in vitro*.

The events transpiring in animal models can be divided into several stages. After infection with wild-type viruses, virus replication ensues in the tissues at or near the site of inoculation. Under normal conditions, this initial multiplication probably ensures contact with and entry into the sensory nerve endings. In animal models, perhaps because of the huge inoculating doses normally used, this step is not required; even mutants totally deficient in replication capability are able to establish latent infections following corneal inoculation (306). The capsid is transported to the neuronal nucleus (331,378). Studies on neurons cultured *in vitro* indicate that the viral capsids are transported by retrograde axonal transport involving microtubules; drugs that disrupt neuronal microtubule structures, or that are known to inhibit retrograde transport of certain compounds, also inhibit the ability of the virus to move from the peripheral endings of neurons to the nuclei (331). Electron microscopic studies indicate that, in neurons infected in cell culture, the viral particle being transported is the unenveloped capsid (378).

In animal models, there is a short period of viral replication in the ganglia at this stage (312,314–317,407,408, 512,513,698,697,711,739). It is not known whether this also occurs in humans or, as seems more likely, is an artifact due to the animal system, route of inoculation, induction of host *trans*-activating factors, or the large amount of virus used in the inoculum to attain a high percentage of latently infected ganglia. For example, corneal scarification, discussed below, has been reported to induce a number of host *trans*-activating factors and viral genes (ICP4, VP5) in neurons harboring latent virus (699).

In the second stage, at most 2 to 4 weeks after inoculation, no replicating virus can be detected in the sensory ganglia innervating the site of inoculation.

In the last stage, certain stimuli (e.g., physical or emotional stress, peripheral tissue damage or intake of certain hormones in humans, and both peripheral tissue damage and administration of drugs that stimulate prostaglandin synthesis in experimental animals), may result in activation of virus multiplication concurrent with axonal transport of the virus progeny, usually to a site at or near the portal of entry. Although the issue is still being debated, the sum of all of the data currently available strongly suggest that virus multiplication results in destruction of the neuronal cell.

Role of Viral Multiplication at the Portal of Entry in the Establishment of the Latent State

There are several important facets of latent infections which relate to the role of virus multiplication, both at the portal of entry and in the neurons harboring the virus.

(i) As noted above, HSV must have access to the nerve endings in order to establish latency, and therefore it could be expected that the greater the number of peripheral cells that become infected and support virus multiplication is, the larger the number of neurons

which will harbor latent virus. The relevant phenomenon in humans is that the frequency of reactivations resulting in recrudescences of lesions is related to the severity of lesions caused by the first infection. In the model we have proposed below, the frequency of recurrences would be determined in part by the number of neurons harboring virus. However, in animal models, there is no absolute requirement for peripheral replication. Viruses lacking the essential α4 and α27 genes, totally unable to replicate in any cell *in vivo,* were shown to be able to establish and maintain latent infections (304,609).

(ii) Many years ago, it was proposed that the latent virus makes a "round trip," i.e., the reactivated virus reestablished the latent state by infecting the nerve endings of hitherto uninfected neurons (313). Currently, available evidence does not support this hypothesis. First, it is very difficult to superinfect in experimental systems ganglia harboring latent virus with a second, marked virus (423). Perhaps even more significant, the "round trip" does not appear to take place in humans even under conditions which would favor such a phenomenon. Thus, in a small number of individuals, mutations which were virulent and acyclovir resistant have arisen (164,477). Recurrent lesions which emerged after the mutant was eliminated with the aid of other drugs did not contain the acyclovir-resistant virus. While this phenomenon has been attributed to rapid elimination of the peripheral infected cells by the immune system (82), the observations may have more profound implications, inasmuch as induction of latent virus should eliminate it from the ganglion.

Viral Gene Expression in Latently Infected Neurons

Extensive studies on ganglia harboring latent HSV have been rewarded by an extreme paucity of evidence for viral gene expression. The only transcript detected to date is one designated optimistically as the LAT (663). This transcript is abundant and accumulates in the nuclei of neurons of latently infected animals and humans (330,545,659,663, 664). The transcript, originally identified as an abundant, nuclear RNA (664), is now believed to be a stable intron (178), approximately 2 kb in length, derived from a primary transcript of approximately 8.3 kb (434,757). The intron itself can be spliced (708,719). The LAT RNAs are conserved in a very similar form in HSV-2 (433). It is difficult to believe that these viruses have conserved throughout evolution the expression of a transcript with no function. However, the function of LAT during latency remains elusive. Confounding the issue are the accumulated masses of data indicating that LAT is not absolutely required for the establishment or maintenance of latency (251,608, 658) or for reactivation of latent virus (48,250,357,696). Experiments must, therefore, be designed to measure small differences in reactivation rates or frequencies, which can be difficult to reproduce. There have been conflicting reports on whether expression of LAT during latency affects reactivation (40,250,357,696); however, in the absence of data on the number of neurons actually harboring latent virus, it is difficult to determine whether this decrease is due to a true defect in reactivation or to an earlier defect in peripheral replication or establishment of latency.

Because it runs in part antisense to the 3′ terminus of the α0 gene, it has been postulated that the function of LAT is to preclude the expression of α0 (664). In this case, however, it would seem that the absence of LAT would allow expression of α0 mRNA, and this does not appear to be so. It has also been postulated that either the primary, polyadenylated transcript or the stable intron may encode a protein and that the function of LAT, whatever that may be, is carried out by this protein. To date, numerous attempts by several laboratories to identify a protein encoded by LAT and expressed during latency *in vivo* have failed. As noted above, antibodies to a peptide homologous to one open reading frame within the nonpolyadenylated intron sequence have been shown to react with a protein expressed during nonproductive infection of sensory neurons in culture; in this case, however, the reactivity of the antiserum with the sensory neurons *in situ* has not been reported (147).

Confounding a total rejection of viral functions expressed for the establishment or maintenance of the latent state is the recent discovery of an open reading frame antisense to $\gamma_1$34.5 and which expresses a protein (346). The transcript was detected late in infection and in nuclear run-off experiments, but not in cells treated with cycloheximide from the time after infection (53,751). However, tagged ORF P protein was detected in cells infected and maintained at 37°C in the present of cycloheximide and then allowed to make proteins in the presence of actinomycin D (346a). The transcript or the protein it encodes has been detected early in infection in cells infected with $\alpha 4^-$ virus (751) or at nonpermissive temperature with mutants in the α4 gene (346). Although attempts to detected the transcript in latently infected neurons have been unsuccessful (751), the results remain inconclusive since both the transcript and the protein are made in very small amounts, and Yeh and Schaffer (751) failed to detect the 8.3-kbp LAT which contains the sequences contained in the ORF P transcript. An added complication is that ORF P and the $\gamma_1$34.5 open reading frame are virtually superimposable, albeit antisense to each other. At least with respect to HSV-1, mutations in the $\gamma_1$34.5 gene result in a decreased incidence of latency and reactivation in the murine model (728).

One of the central issues of HSV latency is why there is no transcription in sensory neurons of promoters expressed in a wide variety of other cell types, particularly the α genes. As discussed in the section on regulation, transcription of α genes is induced by a combination of host and viral factors. It is conceivable then that the lack of α gene expression in sensory ganglia could be due to (a) a

lack of the viral α *trans*-inducing factor, (b) a lack of host factor, (e.g., Oct-1) required for αTIF function, or (c) a direct repression of the α gene promoters, as opposed to a lack of induction. The first possibility was tested using a recombinant virus that contained an insertion of a second copy of the αTIF gene under the control of the mouse metallothionein promoter (604). Despite expression of the chimeric αTIF gene in latently infected sensory neurons, latent infection continued. Furthermore, expression of the same chimeric gene in transgenic mice also failed to prevent establishment or maintenance of latency. *In situ* hybridization with probes specific for Oct-1 demonstrated detectable levels of Oct-1 mRNA in the sensory neurons of the MT-αTIF transgenics (V. Hukkannen and B. Roizman, unpublished data), indicating that the presence of both αTIF and Oct-1 was insufficient to prevent latent infection. Similar studies on the α0 and α4 genes have also failed to block the establishment of latency (R. Fawl, V. Hukkannen, and B. Roizman, in preparation).

Another series of experiments has shown that corneal scarification, commonly used in inoculation, causes induction of transcription of several viral genes and host *trans*-activating factors (Oct-1, c-*jun,* and c-*fos*) (699). At this point, a cause-and-effect relationship between the upregulation of one of these factors and the reactivation of latent virus has not been defined, but is an intriguing possibility.

Copy Number of Viral DNA in Latently Infected Neurons

In a different category from LAT is the observation that, in trigeminal ganglia harboring latent virus, there are between 0.1 and 1 viral genome equivalents per cell genome (65,515,543,544). This datum poses an intriguing question. Heretofore, the number of neurons harboring virus was thought to be between 0.1% and 3% of the total neurons. A recent report by Rodahl and Stevens (546) indicates that, under some circumstances, the number can be higher; however, neurons account for only about 10% of the total cells in a sensory ganglion. Unless viral genomes are contained in every single cell in a ganglion, including glial cells, it is obvious that, as we first calculated a number of years ago (566), each latently infected neuron must contain more than one viral genome.

Two series of experiments indicate that, as we have previously predicted (566), replication of viral DNA by viral enzymes is not necessary for the establishment of latent infections and is not responsible for the attainment of the high copy number. In these experiments (629,645,646), mice were infected by the footpad route, and assays of LAT, lytic antigens, and DNA copy number were done. Although there was a correlation between levels of lytic replication and the level of viral DNA per LAT+ neuron, even those ganglia which showed little or no signs of viral antigen expression still had DNA copy numbers of greater than 20 viral genomes per LAT+ neuron. To account for the high number of viral genomes per cell harboring latent virus, it is then necessary to postulate that (i) more than one viral genome can enter a single neuron during the establishment of latency or (ii) viral genomes are amplified by the cellular machinery during latency (566). In support of the second of these hypotheses, a host-dependent origin of DNA replication embedded within the viral genome has been identified (607).

Viral Gene Expression Required for the Maintenance of the Latent State

To date, no gene or sequence tested, including several essential for lytic viral replication, has been identified as being essential for maintenance of latency. These include all of the genes discussed above under virulence, as well as α0, α4, α27 (358), α47, U_S9-11 (421,459), and U_L24 (606).

Reactivation of Virus from Latent State

The original operational definition of latent virus was dependent on reactivation, i.e., that virus which could be detected after incubation of intact ganglionic tissue with suitable susceptible cells, but not by inoculation of the susceptible cells with macerated ganglia (662). This definition obviously also included the ability to reactivate and replicate to detectable levels in the ganglia. More recently, the definition of latency has come to be extended to include viruses that can be detected in sensory ganglia several weeks after infection by *in situ* hybridization with probes for LAT or by assays of viral DNA in the ganglia (104,304,358,359,379,688). This has allowed assessment of the roles of specific genes in the establishment and maintenance of latency, separate from their roles in reactivation. However, the evidence that reactivatible latent virus can be found under some circumstances in the absence of detectable LAT (546) indicates that these assays must be interpreted cautiously. At the present time, the only technique for proving that a virus is incapable of establishing latent infections is the inability to detect viral DNA by PCR assays of DNA extracted from whole ganglia. It seems likely that a new standard will be the identification of neurons containing viral DNA by *in situ* PCR (165,654).

In humans, latent virus is reactivated after local stimuli such as injury to tissues innervated by neurons harboring latent virus or after systemic stimuli such as physical or emotional stress, menstruation, hormonal imbalance, and so forth which may reactivate virus simultaneously in neurons of diverse ganglia (e.g., trigeminal and sacral). In experimental systems, multiplication of latent virus has been induced by physical trauma to tissues innervated by the neurons harboring virus (13,233), by iontophoresis of epinephrine (339,340) or other drugs (223,234,623), by transient hyperthermia (592,593), and by corneal scarification (699). Recent studies showed that injection of cadmium

resulted in reactivation of a HSV-1 in a fraction of neurons harboring latent virus and that this reactivation was not associated with induction of the metallothionein genes, inasmuch as other injections of other heavy metal solutions at much higher amounts failed to induce viral reactivation (179). The hypothesis advanced to explain these results is that cadmium specifically inactivates a repressor.

The molecular basis of reactivation and the order in which viral genes are induced is not known. Attempts to identify genes specifically required for reactivation have implicated two genes specifically in reactivation events, i.e., ICP0 and LAT, although in both cases conflicting data have been published (48,101,224,248,357,358,696). However, as outlined above in the case of LAT, the absence of data on the number of latently infected neurons from which the virus is being reactivated makes these data difficult to assess. Particularly in the case of ICP0 deletion mutants, the attenuation of virus growth in all tissues makes it very hard to say that the gene plays a reactivation-specific role. Virtually all the recombinant viruses described in the Virulence section of this chapter that are attenuated for growth in peripheral and CNS tissues also exhibit reduced reactivation rates by explant cultivation of latently infected sensory ganglia; this makes it difficult to attribute any reduced reactivation frequency to a reactivation-specific role.

Modeling the Latent State

The molecular basis of latency rests on answers to several key questions. First, since HSV readily multiplies in a variety of cells derived from human or animal tissues, why does lytic infection not ensue in neurons harboring latent virus? Second, at what stage in the reproductive cycle is viral multiplication arrested? Third, what is the origin of the high copy number of the viral genome in latently infected neurons? Fourth, why does the virus not reactivate from all neurons at the same time? Fifth, at what stage in the cascade of viral gene expression does replication of reactivating virus begin? Sixth, why is HSV-2 more readily reactivated from sacral ganglia, whereas HSV-1 is more readily activated from trigeminal or cervical ganglia?

Our model, presented largely for its heuristic value, is based primarily on the hypothesis that latency is required for the perpetuation of the virus in its natural host population and that the virus has evolved elaborate mechanisms to control the latent state. It is a further modification of a model that we have proposed in the past (58,565) and consists of several components as follows.

1. Inasmuch as expression of αTIF plus Oct-1 in latently infected neurons was not able to induce expression of lytic replication, it appears that the lack of expression of α genes must be due to either repression, a lack of other host proteins involved in α gene transcription, or both. Repression could be caused by a viral or host-encoded protein binding to some sequence in the vicinity of α gene promoters, or it could be due to a DNA conformation effect (the two are not mutually exclusive). Either of these modes of repression could potentially be relieved by replication of the viral genome. Obviously, a requirement for DNA replication for expression of HSV genes is not unique to this situation but is directly analogous to the regulation of γ genes.
2. The model also proposes that activation of virus multiplication is the consequence of the cumulative effect of stimuli to which each cell harboring latent virus responds independently. Specifically, the hypothesis envisions that both local and systemic stimuli cause the latent viral genome to be replicated by host enzymes, utilizing a previously mapped host origin of DNA replication present in the viral DNA (607), thereby causing the copy number to be increased. DNA replication could overcome repression by either diluting a repressor (a copy number effect) or changing the conformation of replicating DNA. As the stimuli encountered by each neuron would be different, the copy number of viral DNA in each latently infected cell would increase independently.
3. The increase in the DNA copy number may not be sufficient to ablate the block in virus multiplication. A second function, e.g., induction of a host transcription factor, could also be necessary. The combination of requirements for both release of repression (by DNA amplification) and addition of a positive factor could explain why latently infected neurons reactivate individually, rather than as a group.
4. Additional requirements for viral multiplication during reactivation fall under the heading of capacity for gene expression and are poorly defined. A common feature of this property is seen in the case of deletion mutants (e.g., $\alpha 22^-$) (603) infecting nonpermissive or restrictive cells. Such mutants often exhibit a multiplicity dependence reflected in the failure of virus multiplication at low, but not at high, multiplicities of infection. The additional functions that may be required to achieve clinically detectable reactivation (or detectable amounts of infectious virus in experimental systems) may be those of a set of viral genes. The model proposes that (a) this set would include genes that are required as well as those that are dispensable for multiplication in cells in culture and (b) the expression rate and product abundance of these genes would determine whether infection is productive or abortive in the particular cell in which the virus is latent.

A similar model of HSV latency and reactivation has recently been proposed by Kosz-Vnenchak et al. (326). These authors have also proposed that DNA replication may be a critical stimulus during reactivation of latent virus. However, they propose that the replication is taking place via the normal viral enzymes, expressed as β genes. In their experiments, mice were infected with wild-type virus or a

tk⁻ viral mutant, and after the establishment of latency ganglia were explanted and incubated for 48 hr. *In situ* hybridization indicated that expression of viral α and ß genes was much lower in ganglia from mice infected with the *tk*⁻ virus than in those containing wild-type virus. The authors have proposed that the ß genes involved in DNA replication, including *tk*⁺, are required for the initial stages of reactivation by increasing the expression of α genes to a level sufficient to stimulate lytic infection. In this case, the high DNA copy number during latency is accounted for by sporadic low-level expression of the viral replication enzymes. The significance of this model stems from the authors' conclusions that DNA replication somehow stimulates α gene expression and further that the mechanisms of gene expression defined in cells in culture do not hold true for sensory neurons. In these experiments, very few sections were examined for each ganglion, and the number of LAT⁺ neurons per ganglia were not assayed prior to explant, leaving open the possibility that the wild-type virus had spread within the ganglia and that the positive signals were coming not only from cells that had initially contained latent virus. The γ gene products in wild-type infected ganglia were not detectable under these conditions until 48 hr postexplant; there may have been a sensitivity problem in these studies in that experiments by a different group of investigators have shown that, following explant of wild-type latently infected ganglia, transcripts of all classes of genes (α, ß, and γ) can be detected by PCR analyses as early as 4 hr postexplant (143). This very quick induction of viral gene expression following explant indicates that, by 48 hr, considerable virus spread within the ganglion may have occurred.

SIGNIFICANCE OF THE NUCLEOTIDE DIVERGENCE OF HSV-1 AND HSV-2

It can be argued that the differences in the nucleotide sequences of HSV-1 and HSV-2 predicts appropriate differences in the cells lining the oral and genital mucous membranes. The data described above for gC-1 and gC-2 support this hypothesis. In addition, the differences in rates of reactivation of HSV-1 and HSV-2 from trigeminal and sacral dorsal root ganglia may also reflect differences in viral functions required for lytic growth in each cell type.

VIRAL MODULATION OF HOST DEFENSE MECHANISMS

Like other large DNA viruses, HSV appears to have evolved a variety of mechanisms of evasion of the host defense machinery. They include genes that prevent apoptosis in differentiated cells, that interact with antibody and complement, and that prevent induction of $CD8^+$ cytotoxic T cells.

The association of the $\gamma_1$34.5 gene with neurogrowth is an excellent example. As noted above, several laboratories have associated loss of the capacity to cause death by intracerebral inoculation of mice with mutations in sequences in the right end of the L component (282,283,577,681,682, 689,690). Independently of these studies, a gene mapping in the repeats of the L component and designated as $\gamma_1$34.5 (5,93,95,97) was found to play a major role in the ability of the virus to replicate in the CNS, inasmuch as mutants lacking a functional $\gamma_1$34.5 gene exhibited $>10^7$ pfu/LD_{50} by intracerebral inoculation even though the virus is capable of limited replication on intravaginal inoculation of mice (728). Because of these results, further studies were carried out in a variety of cell lines with the following results.

1. The $\gamma_1$34.5 gene is dispensable in some cell lines (e.g., Vero) and the ability of the mutant virus to replicate cannot be differentiated from that of the wild-type virus (5,91,97). In the human neuroblastoma cell line SK-N-SH and in other cell lines of neuronal origin, deletion mutants and stop codon mutants in the $\gamma_1$34.5 gene trigger a premature total shut off of all protein synthesis, thereby rendering the cell nonviable and drastically reducing viral yields (94). The stress response which leads to the termination of synthesis is triggered by an event associated with viral DNA synthesis, inasmuch as exposure of cells to PAA, an inhibitor of viral DNA synthesis, precludes the premature termination of translation (94). It is noteworthy that, although protein synthesis is shut off, viral DNA accumulates to near-normal levels, and the presence of γ_2 mRNA late in infection argues against a role of the viral *vhs* gene in this process.
2. The HSV-1(F) 263-amino acid $\gamma_1$34.5 protein consists of a 159-amino terminal domain, ten repeats of the amino acids AlaThrPro, and a 74-amino acid carboxy-terminal domain (95). The repeat varies from strain to strain (five to ten repeats) and may be considered a linker sequence (95). Cellular homologs identified to date include MyD116, a 657-amino acid protein expressed in myeloid leukemia cells induced to differentiate by interleukin-6, and GADD34, a protein induced by growth arrest and DNA damage. These proteins also contain a long amino-terminal domain separated from a shorter carboxy-terminal domain by a repeat—in these instances, a 38-amino acid sequence repeated 4.5 times. The carboxyl terminus of $\gamma_1$34.5 is homologous to those of Myd116 and GADD34. Analyses of viral mutants from which various domains of the $\gamma_1$34.5 gene had been deleted or rendered mute by the insertion of a stop codon have shown that the carboxyl terminus of $\gamma_1$34.5 protein, i.e., the sequence shared with Myd116 and GADD34, is required in order to preclude the stress response which leads to total shut off of protein synthesis (93).
3. A surprising finding was the discovery that in human foreskin fibroblasts, the shut off of protein synthesis was triggered by a deletion mutant lacking 1 kbp of

coding sequence but not by the stop codon mutants in human foreskin fibroblasts. This observation dispelled the notion that the $\gamma_1$34.5 was required exclusively in neurons but raises the issue as to why stop codon mutants behave like null mutants in human neuroblastoma cells but not in human foreskin fibroblasts. One clue is based on the observation that very small amounts of $\gamma_1$34.5 are required to block the stress response which leads to shut off of protein synthesis. It is conceivable that there is a low-level suppression of the stop codon in human fibroblasts or that the amounts required to block the stress response are lower in fibroblasts than in the neuroblastoma cells (92).

The studies on the $\gamma_1$34.5 gene mutants indicate that at least some host cells respond to the stress of HSV infection triggered by the onset of viral DNA synthesis. This response results in complete shut off of protein synthesis and, invariably, from the point of view of viral infection, a premature death of the infected cell. It is an important response, inasmuch as it curtails the ability of the cell to support viral replication and spread throughout the body. The inability to prevent the cellular stress response which causes the infected cell to die may be responsible for the inability of the deletion mutant to multiply and cause pathology in the CNS of mice. The virus has evolved a gene, possibly borrowed in part from eukaryotic cells, to preclude this response in order to replicate in a variety of infected cells.

Herpes simplex virus type 1 expresses two types of IgG Fc receptors on the cell surface (34,152,473,474). One, a complex of gE and gI (288), binds monomeric IgG, while the other, consisting of gE alone, binds polymeric IgG (152). The Fc receptors appear to play a role in protecting infected cells or virions from the host antibody response. Cells in culture infected with wild-type virus are relatively resistant to antibody-dependent cellular cytotoxicity as compared to cells infected with a gE deletion mutant (152). Because of the multifunctional nature of gE and gI, studies to determine the role of this protection in pathogenesis will have to await specific mutants that eliminate the Fc binding functions without altering their functions in the viral entry into and egress from infected cells.

The second component of the immune system known to interact with HSV proteins is complement. The complement component C3b has been shown to bind to both gC1 and gC2 (420,610). Mapping of the domains of the viral glycoproteins involved in binding C3b has indicated that several domains are necessary, including one that has some homology to the cellular C3b receptor, CR1 (269). Here again, because of the role of gC in attachment to some cell surfaces, specific mutations that do not affect attachment to polarized cells must be made to determine whether gC fulfills its predicted role in protection of the virus from the host complement system *in vivo*.

Herpes simplex virus also appears to protect itself from a strong host $CD8^+$ cytotoxic T-cell response. It has been known for a number of years that, in humans, epithelial lesions caused by HSV contain a disproportionate number of $CD4^+$ and very few $CD8^+$ T cells (120,596). Experiments in which HSV-infected human fibroblasts were exposed to human anti-HSV cytotoxic T lymphocytes (CTL) indicated that infection actually caused the fibroblasts to become resistant to lysis (497). This resistance phenotype was mapped to the right end of the S component (80). Later studies have shown that expression of sequences containing the α47 gene causes a resistance to lysis by $CD8^+$ CTLs, due to retention of the MHC class I molecules in the cytoplasm and a lack of peptide presentation on the cell surface (754). ICP 47 has recently been shown to interact directly with the transporter associated with antigen processing (TAP), inhibiting peptide translocation into the ER in human but not murine cells (Zola, 247a).

Herpes simplex virus also appears to have a second function capable of inactivating $CD8^+$ T cells that come into contact with an HSV-infected fibroblast; in this case, the viral gene products responsible for this activity have not been identified (496,753).

CONCLUSIONS

We permit ourselves a personal comment. The field has grown enormously since the first attempt to understand the biology of the virus was undertaken by one of us more than a third of a century ago. The studies on HSV are at last entering a most exciting period largely because the words "structure and function" are beginning to have an operational meaning. As a field of endeavor, we are beginning to characterize the interaction of proteins among themselves and with viral nucleic acids. In addition, the host factors crucial to virus multiplication and, potentially, to latency are being sought out. The armamentarium for a major assault on the mysteries underlying the biology of these viruses is in place and reflects the contributions of many laboratories over many, many years.

At the same time, the field has grown too much. The task before us was far greater for not having followed Ludwig Wittgenstein's dictum that "Whereof one cannot speak, thereof one must be silent," but chapters of citations bereft of commentary are dull. We fear that the commentary of the future will be significantly restrained for lack of both space and capability to deal with the enormous windfall of information that only a genome of 150,000 bp can produce.

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